Influence of biotic and abiotic factors on the composition and function of a 4-chlorosalicylate degrading consortium

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1 Introduction

1.1 Problematic substances: xenobiotics

Synthetic organic substances were produced throughout the world due to their widespread use as agricultural chemicals, detergents and plastics. Disposal of industrial wastes, leakage and volatilisation has led to the release of these xenobiotics (synthetic products not formed by natural biosynthetic processes) into the environment. These organic pollutants can lead to damage of plants and animals in polluted areas and they can cause health problems and genetical defects in humans. Waste disposal sites and chemical accidents are sources for the accumulation of pollutants in food chains and for air and drinking water pollution. These sites have to be cleaned up to reduce the negative influences of the pollutants upon the environment and the human beings. Conventional technologies involve removal, alteration, or isolation of the pollutant. These technologies typically consist of excavation followed by incineration or containment. Such conventional treatment is cost extensive and often results in the transfer of the contaminating compounds from one environment or form to another. Bioremediation is the result of the biological breakdown, or biodegradation, of contaminating compounds. Biodegradation can be defined as the breakdown of organic compounds, usually by microorganisms, into more cell biomass and less complex compounds. Some organic pollutants can be mineralised to CO_2, H_2O and other inorganic components, whereas others are recalcitrant (poorly biodegradable) and persist in the environment. Bioremediation is an attractive alternative to physicochemical methods or disposal, as it is cheap, can result in mineralisation of organic pollutants and can be used *in situ* for the cleanup of low, but environmentally relevant concentrations of pollutants. The mineralisation of complex materials into simple inorganic constituents is important for the recycling of carbon, hydrogen, oxygen, nitrogen and sulphur.

1.2 Microorganisms in biodegradation: Pure cultures and consortia

Microorganisms are especially useful for bioremediation because of their great metabolic diversity. In the cycling of material play mostly heterotrophic microorganisms the role of decomposers. Therefore these organisms are partly able to mineralise pollutants and synthetic organic substances, which resemble in their structure natural substances, such as petroleum and chlorinated hydrocarbons. This ability of the microorganisms was made use of in biodegradation applications. Biodegradation studies have been based on classic procedures which result in the isolation of an organism able to grow on the desired compound.

1.2.1 Pure cultures

Traditionally, the activities of microorganisms relevant to biodegradation have been studied in pure monocultures. Enrichment cultures were followed by selection with the aim to isolate a pure culture with the capacity to use the required compound as growth substrate. Biodegradation mechanisms were determined by elucidating the catabolic sequence, purifying and identifying intermediate metabolites, assaying and characterising the enzymes involved in the pathway and determining the factors controlling the regulation of pathway expression. These approaches have provided a deeper insight into the individual metabolic pathways and underlying mechanisms. These pure culture studies revealed that (among others) two main criteria influencing the degradability of xenobiotics are the structure of the pollutant and the adaptation and genetical composition of the microorganisms.

Some microorganisms are able to degrade xenobiotics which have structural resemblance with naturally existing substances. These substances are unsubstituted mono-, or di-cyclic hydrocarbons. The microbial degradation of aromatic hydrocarbons decreases with the increasing number of aromatic rings of the aromatics and with the existence of substituents (halo-, nitro- and sulpho-groups) (Commandeur & Parsons, 1994; Reineke, 1984). Some representatives of this class of compounds with low toxicity such as chlorobenzoates or chlorophenoxyacetates are totally degraded by single organisms (Chatterjee et al., 1981; Kim & Picardal, 2001). Other substances such as polychlorinated biphenyl (PCB), organic solvents, dioxins, or higher substituted aromatics are highly complex in their chemical nature and are recalcitrant (poorly biodegradable) due to their specific structural skeletons and substituted groups or high molecular weight (Atlas & Unterman, 1999).

The ability of microorganisms to degrade pollutants can be enhanced. One possibility is prolonged adaptation of mixed cultures in chemostats under high selective pressure. Such a procedure has led to the isolation of haloaromatics degrading bacteria (Dorn et al., 1974; Hartmann et al., 1979). The molecular mechanisms of genetic adaptation to xenobiotic compounds were reviewed by van der Meer et al. (1992).
Another possibility to enhance degradative abilities of microorganisms is genetical engineering. The transfer of genetic elements from one microorganism to another with the view of broadening the spectrum of degradable compounds or to ensure their inclusion into the catabolic route of the recipient strain was achieved in experiments using conjugation, transduction or transformation (Boronin, 1991; Boyle, 1992; Reineke & Knackmuss, 1978). The first genetically engineered microorganisms (GEMs) of this type were constructed 1984. The experiment resulted in the expansion the catabolic range of the microbial strain to include chlorosalicylates (Lehrbach et al., 1984). In another example was the recalcitrance of chloroaromatics based on the fact that degrading strains only inherited one pathway, while the other pathway, necessary for the complete degradation of the substrate, has been missing. The

omission of the “lower” pathway resulted in the accumulation of chlorinated catechol (Wilkes et al., 1996). By "patchwork assembly", so is the strategy called, to complete missing sequences in a degradation pathway of one strain by the combination of genes of different bacterial strains, complete degradation of some pollutants could be achieved (Timmis, 1994).
Although these approaches have led to immense achievements, there are still recalcitrant pollutants in the environment. For example higher condensated chloroaromatic substances are mainly co-metabolically transformed (Commandeur & Parsons, 1994; Horvath, 1972). The recalcitrance of the remaining pollutants is usually based on the chemical stability of the structural elements of the chemicals, or on toxicity for microorganisms, or on the inhibition of degrading enzymes (Ramos et al., 1994).

Pure culture studies provided a wealth of knowledge of basic principles, degradation pathways and involved enzymes and their regulation. They have led to successful application of microbial strains in industrial processes, for example in reactors or in “end of pipe” applications. The studies highlighted the relationship between one degrading organism and the chemical of interest. But in nature, most environments support the growth of a wide range of microorganisms having many different metabolic capabilities. Relationships and interactions occur between organisms, which grow in close proximity to each other. It has been shown that these relationships between the populations result in beneficial effects which make the associations more successful than any of the individual populations alone.

1.2.2 Consortia

In the field of biodegradation, the cooperation between different microorganisms is of specific interest because synergistic (both populations benefit from the relationship) interactions occur. Although undefined communities (all of the organisms that occupy a particular site, as defined by (Alexander, 1997)), which are treated as integral biocatalytic "back boxes", have allowed us to handle, in many instances, contaminated streams or sites, the lack of knowledge of the interrelationships among community members as well as of community function regulation and control often resulted in unexplainable failures or malfunctioning of the treatment systems. In most cases to date, bioremediation has depended on the ability of naturally existing microbial communities to degrade hazardous waste chemicals under environmental conditions that have been managed to enhance their activity. This approach has worked successfully for certain types of contaminants under a variety of site conditions. Treatable wastes include petroleum products, creosote and non-chlorinated solvents. One solution to enhance bioremediation of xenobiotics in the environment is to make use of microbial consortia (a collection of organisms that have some functional association with each other).
Examples of cultured consortia include heterotrophic-photorophic associations (Schiefer & Caldwell, 1982), interspecies hydrogen or formiate transfer (Bryant et al., 1967; Fernandez et

al., 2000), food-chains and degradative consortia. An example of sulphur-cycling associations is the *Chlorochromatium aggregatum* consortium (interaction between a sulphate reducer and a sulphate oxidiser) (Pfennig, 1980). A recent study concerns the anaerobic methane oxidation by a marine microbial consortium (Boetius et al., 2000). Many microbial processes are only possible due to these consortia e.g., industrial fermentations (such as vitamin B12 production or acetic acid production) (Harrison, 1978) or biotechnological applications (Zeikus & Johnson, 1991).

In many situations, a network of microorganisms is required for complete degradation of refractory molecules or to stabilise the degradation process. Often a community or consortium performed better than pure cultures, both in attaining maximal cell densities and in removing the pollutant. For example, substrate mixtures present in industrial waste streams and contaminated waste sites often require activity by mixtures of microorganisms to be completely mineralised. More examples are listed in Table 1.

Table 1: Examples of consortia completely degrading pollutants

pollutant/substrate	**consortium/community composition**	**reference**
fluorobenzene	two S*phingobacterium/Flavobacterium*; one *Alcaligenes* species	(Carvalho et al., 2002)
3-chlorobiphenyl	*Burkholderia* sp. LB 400; *Pseudomonas* sp. B13(FR1)	(Nielsen et al., 2000)
monochloro-dibenzofuran	*Sphingomonas* sp. RW16 and *Pseudomonas* sp. RW10	(Wittich et al., 1999)
nitrate esters	*Arthrobacter ilicis* *Agrobacterium radiobacter*	(Ramos et al., 1996)
herbizide dicamba (3,6-dichloro-2-methoxybenzoic acid)	*Pseudomonas paucimobilis* *Achromobacter* sp. *Flavobacterium* sp.	(Fogarty & Tuovinen, 1995)
4-aminobenzene-sulfonic acid	*Hydrogenophaga palleronii* *Agrobacterium radiobacter*	(Feigel & Knackmuss, 1993)

There are various reasons for the degradation ability or enhanced degradation ability of consortia. De Souza demonstrated that a consortium consisting of four or more bacterial species, of which two members were *Clavibacter michiganese* ATZ1 and *Pseudomonas* sp. strain CN1, could metabolise atrazine faster than did *C. michiganese* individually (De Souza et al., 1998). The more efficient degradation was based on the degradation of metabolites (alkylamines), which prevented a pH increase (due to amine accumulation). Ambujom studied the composition and stability of a consortium degrading phenol, which consisted of 10

members (Ambujom, 2001). Combined metabolic processes and lack of accumulation of degradation intermediates maintained the stability of the system.
The different mechanisms can be summarised in classes of interactions observed in microbial communities degrading xenobiotics (Slater & Lovatt, 1984):

1. Provision of specific co-factors/nutrients;
2. Removal of toxic products;
3. Modification of growth parameters;
4. Concerted metabolism;
5. Co-metabolism;
6. Gene transfer.

In summary, some xenobiotics, due to their chemical composition, are difficult to degrade. Undefined communities have often been successfully used to detoxify of contaminated streams or sites, depending on the degradability of the pollutant. Pure cultures, which consist of adapted natural isolates or of genetically engineered microorganisms, were used to inoculate the contaminated soil or water in other cases. The study of degradative abilities of defined consortia has revealed that these benefit in different ways from the interactions of their members.

But, although in many cases the difficulties between the target chemical and the microorganisms could be overcome, sometimes the degradative function of the microorganisms was not realised, or the efficiency lagged behind expectations. In many of these cases, the influence of ecological conditions were not known and probably hindered degradation. To develop remediation strategies, the relationship between microbial population dynamics, changes in substrate concentrations and the parameters influencing the consortium behaviour must be understood. It is essential to identify the parameters which govern consortium (and community) structure and function.

1.3 Environmental and stress factors, altering community structure and activity

> "Microorganisms are not static, independent objects that can be adequately understood through isolation. Each is part of a dynamic ecological network that optimises the utilisation of environmental resources (conversion of abiotic resources to biotic resources). If the mechanisms of these interactions are to be understood, then the community must be sustained, cultivated, and studied as a causative agent under controlled laboratory conditions-... If the design theory of life is to be understood, the relationship between environmental stress, reproductive success and the expression of each trait must be determined experimentally. In the same sense, it is not enough to know the species composition of a community, it is essential to also know the relationship of each organism (and each of its traits) to the formation, reproductive success, efficiency, internal interactions and habitat range of its community. It is also

> necessary to elucidate these relationships in experimental laboratory studies under defined environmental conditions."
> (Caldwell et al., 1997)

The success of bioremediation processes is determined not only by the relation of the pollutants with the pollutant-degrading microorganisms, but also by the interaction with other microorganisms and with the physicochemical environment. Recent work focussed mainly on the optimisation of interactions between the pollutants and the degradative microorganisms. Less attention was given to the study of the interactions between the degradative microorganisms and other microorganisms (and macroorganisms) and the interaction between the degradative microorganisms and the physicochemical environment. Environmental factors and conditions affect the growth and the variety of metabolic activities of different microorganisms differently and hence influence the extent and rate of pollutant-degrading activities. The slow biodegradation of xenobiotics in the environment may be caused by unfavourable physicochemical conditions, (such as temperature, pH, ionic strength, oxygen concentration, water activity), high concentrations of contaminants, toxic metals or solvents or may be affected by the availability of other nutrients, the accessibility of the substrates (solubility, dissociation from absorbed materials) (Paerl, 1998; Yaganita, 1990), or predation e.g., (Goldstein et al., 1985; Swindoll et al., 1988).

Among all these factors, physical and chemical factors, such as alternative carbon sources (Bauer & Capone, 1988; Egli, 1995), temperature (Fey & Conrad, 2000), oxygen (Bradshaw et al., 1996a), nutrients (Bogan et al., 2001; Harkness et al., 1993; Steffensen & Alexander, 1995; Wolfaardt et al., 1994), salinity (Kästner et al., 1998; Shiaris, 1989), water activity, metal ions (Baath et al., 1998) and pH (Kästner, et al., 1998) have been identified as the main factors influencing community structure and/or biodegradation. Additionally, biological factors have an influence based on the interactions of microorganisms (or other biological components). Competition and predation are two factors which are relevant in biodegradation processes. Competition, which was studied in this thesis, occurs when two populations are striving for the same resource or niche (Duetz et al., 1994).

In this work, the following factors were selected to be studied: the influence of additional carbon sources, the influence of oxygen reduction and the influence of competition with optimised strains. The reasons for the selection of these factors are explained in more detail in the next two sub-chapters.

1.3.1 Abiotic factors

Mixed substrate utilisation and the influence of reduced oxygen concentration were selected as factors to study the reaction of the consortium. The utilisation of mixtures of substrates by

mixed microbial populations is a subject that is relevant to research in many areas of biotechnology. Biodegradation of a substrate within a contaminant mixture has been shown to be different from the transformation of the compound as a single substrate (Arvin et al., 1989). Often, remediation and waste treatment efforts require the control of the concentration of individual pollutants. For these purposes, it is important to be able to understand and predict the biodegradation kinetics of pollutant mixtures (Senior et al., 1976). Egli states that in ecosystems both the rate and the extent of biodegradation of xenobiotic organic compounds at low concentrations are probably controlled by the presence of other organic compounds (Egli, 1995).

The catabolism of aromatic hydrocarbons by bacteria involves the oxidation of the substrate by oxygenases, for which molecular oxygen is required. For example, levels of catechol 1,2-dioxygenases in strains of *Pseudomonas putida* have been shown to increase with dissolved oxygen tension (Villiesid & Lilly, 1992). Acting as a cosubstrate for oxygenation reactions (e.g., the initial step of the 4-chlorosalicylate degradation) and as a terminal electron acceptor, oxygen is important during aerobic degradation of chlorinated aromatics. Rates of degradation of organic pollutants may become limited by insufficient oxygen (Ghiorse & Wilson, 1988; Hopkins et al., 1993).

1.3.2 Biotic factors

Interactions between microbial populations can include competition for substrates, inhibition (amensalism) by the production of toxins, and enhancement (commensialism) by either the production of growth factors or by the destruction of inhibitory substances. Of the various types of interactions between microbial populations, competition for carbon is often the major determinant of the relative levels of abundance of indigenous organisms in soil environments. The most extensively studied interaction observed between microbial populations is competition for a substrate (Duetz et al., 1994; Steffensen & Alexander, 1995), e.g. Filonov et al. studied competition of plasmid-bearing *Pseudomonas putida* strains catabolising naphthalene via various pathways. They observed that under naphthalene limitation, the strains bearing a plasmid containing the *ortho*-cleavage pathway were the most competitive (Filonov et al., 1997).

Microbial strains can be optimised for the use in biodegradative processes (see chapter 1.2.1). Although there have been some successful experiments with adapted strains (Barles et al., 1979), often the introduced optimised strains fail to survive in the natural environment (Goldstein et al., 1985). In other cases the strains survive, but the degradative activity does not occur or does not last permanently (Boon et al., 2000).

Releases of recombinant microorganisms were summarised by (Bailey et al., 1999; Sayler & Ripp, 2000; Wilson & Lindow, 1993). The field performance of these GEMs (Schwieger & Tebbe, 2000) (and often also the performance in microcosms (Filonov et al., 1999; Nusslein et al., 1992; Watanabe et al., 1998)) has been inconsistent. Even environmentally competent

strains did show loss of ecological competence, poor survival, lack of fitness and inability to compete, based on the biotic interactions with the indigenous microflora, which displays among other interactions competition. In this thesis it was studied if optimised strains have the ability to compete with a consortium.

Consortia (or communities) should be able to tolerate influences and variations of abiotic or biotic environmental factors. Therefore, they should be stable (within certain ranges) and efficient in biodegradation processes.

The influence of these factors on communities degrading pollutants in the field was not often studied in the past. The reason for this exclusion is the quantity and variety of factors influencing the degradation under such conditions and the fact that the habitat is subjected to temporal and spatial changes. Another hindrance was the problem of analysing the structure of communities in the field.
Often studies concern either the influence of changing factors upon the structure of communities, with limited information about their function (for example was the influence of environmental conditions upon species abundance and diversity studied (Atlas, 1984; Chapin III et al., 1997; Naeem et al., 1994)), or upon the function of communities, treating the composition of the community as "black box". For example studied Lay et al. the environmental factors influencing methane production from high-solids organic waste by an unknown seed sludge community (Lay et al., 1997). Threshold and optimum of moisture content, pH and nitrogen supply were shown to influence methane production, without the observation of the influence upon the structure of the community.
Besides the amount and variety of factors and the continuous alteration, was another hindrance to study the influence of these factors on communities degrading pollutants in the field the problem of analysing the structure of communities in the field, as fewer than 1% of the microorganisms present in many environments may be readily culturable (Amann et al., 1995; Kell et al., 1998), and there may be as many as 4,000 species per gram of soil (Torsvik et al., 1990). To overcome those difficulties, on one hand new methods/technologies based on the direct extraction and analysis of indicator molecules such as nucleic and fatty acids were developed (Ogram & Feng, 1997). On the other hand the exact study of the influence of these factors is only possible applying a model system, which can be maintained under conditions which allow the change of one selected factor while keeping other factors constant (see citation from Caldwell at the beginning of this chapter). Another huge advantage of the application of such a model system is the ability to monitor the influence of changing factors upon the structure and the function of the consortium simultaneously. The model system, which was applied in this thesis is introduced in the next chapter.

1.4 The model system: The 4-chlorosalicylate degrading consortium

The model system, which was selected for the study of environmental influences upon consortium structure and function was a four membered consortium, which was maintained in continuous culture with a chlorinated aromatic as sole source for carbon and energy.

1.4.1 The chemostat

Chemostat enrichments provide excellent possibilities for selective enrichment of entire microbial communities (Christensen et al., 2002; Magliette et al., 1996; Stoffels et al., 1998). Balanced growth under sufficient but poor to moderate nutritional conditions, such a microorganisms encounter in the field, can be achieved (Koch, 1997). Classical examples are those in which fermentative bacteria produce secondary substrates, or in which vitamins or other requirements excreted by two species establish commensalistic or mutualistic relationships. These types of interaction may also be studied in batch culture systems, but the significant advantage of doing it in continuous culture is the degree of constancy of the growth conditions that can be obtained, and the avoidance of interfering lag-phases, substrate exhaustion and variable product accumulation, and changes in biomass, characteristic of batch culture experimentation. In particular, the possibility to maintain cells for long periods of time under rigorously controlled environmental conditions permits detailed studies of the way microorganisms respond to particular environmental influences.
Brock claims that the good model system uses well-defined cultures, low-density populations, low nutrient concentrations and slow growth rates (Brock, 1987). The culture of a consortium in a carbon-limited chemostat fulfils these requirements and was therefore applied in this thesis. It mimics in the laboratory a part of the natural environment. The chemostat was used to establish a stable consortium. The continuous culture provided further the opportunity to study the consortium's food web and to apply disturbances. Although a chemostat is an open system, it can be maintained under highly controlled conditions, which allow studies with genetically engineered microorganisms as well. In the next sub-chapter is the selection of the sole carbon source, which was applied in the chemostat, explained.

1.4.2 Consortium composition and history

The consortium was established to study the relationship and interaction of consortium members. A continuous culture system under carbon limiting conditions with 4-chlorosalicylate (4-CS) as sole carbon source was used to enrich the 4-CS degrading consortium. The inoculum of the chemostat was a sediment sample (from the aerobic top layer) of the polluted Spittelwasser, a small tributary of the Elbe River near Bitterfeld, Sachsen-Anhalt, Gemany, which was polluted for decades by untreated waste water from the chemical industry (Wagner-Döbler et al., 1993). Sediment of this river was used before by Thakur, as a source for the enrichment of 4-CS degrading bacteria (Thakur, 1995). Once the

microbial consortium reached equilibrium (Faude, 1996), it consisted principally of four strains, which were characterised by 16S rRNA gene sequencing and comparing with gene databases. The strains have been classified as belonging to the genera *Pseudomonas* (*Pseudomonas* sp. MT 1, *Pseudomonas* sp. MT 4), *Empedobacter* (*Empedobacter brevis* MT 2) and *Achromobacter* (*Achromobacter xylosoxydans* MT 3, former *Alcaligenes* sp.). *P*. sp. MT 1 was identified as the primary degrader (Faude, 1996), due to the fact that this strain was the only one of the four consortium members which could grow in batch culture with 4-CS as the only carbon source.

Studies concerning the 4-CS degrading consortium served the aim to establish a method for the identification and enumeration of the consortium members, perform simple disturbance experiments and unravel the metabolic network. Faude established the immunochemical characterisation of the consortium (Faude, 1996). Pure cultures of the single isolates were used to generate monoclonal and polyclonal antibodies for the determination of specific cell counts by indirect immunofluorescence microscopy (Faude, 1996), colony blots, and immunological fingerprinting (Frech, 1996). Frech performed two perturbation experiments successfully (Frech, 1996).

The stability of the consortium structure indicated that the structure was characterised by the different functional roles of the consortium members in the food-chain generated by the degradation of 4-CS. To unravel the network of carbon sharing in this 4-CS degrading bacterial consortium was the aim of Pelz' Phd. thesis (Pelz, 1999a).

1.4.3 4-chlorosalicylate, a model chlorinated aromatic

A class of compounds of high environmental importance are the chlorinated aromatic compounds. They have been used in agricultural or industrial processes. As halogenated aromatic compounds are usually markedly more refractile to microbial attack than nonhalogenated aromatics (Reineke, 1999), they have to a significant amount accumulated in the environment. Problems arising during the degradation of these substances are the occurrence of only one pathway ("upper" or "lower") in one microorganism and/or in the misrouting of chemicals in unsuitable pathways leading to "dead-end metabolites" (metabolites which can not be degraded further), or "suicide-metabolites" (metabolites, which are toxic to the producing organism) (Pieper & Timmis, 1996; Reineke, 1984). This leads to the accumulation of ecotoxicologic products, which can "kill" microbial communities and inhibit further biodegradation processes. A soluble halogenated aromatic compound, 4-CS, was selected as carbon source for the isolation and maintenance of the model consortium. The solubility makes it an easy treatable model compound. 4-CS is an intermediate in the aerobic degradation of important organic environmental pollutants such as halogenated dibenzofurans (Arfmann et al., 1997; Wilkes et al., 1996) or chlorinated naphthalenes (Schmitz et al., 1995).

1.4.4 Degradation of aromatic and chloroaromatic compounds

Despite the above mentioned problems numerous microorganisms capable to mineralise chloroaromatics have been isolated in the recent decades. The degradation pathways are here shortly introduced, as the members of the consortium inherit the enzymes for these pathways. Depending on the bacterial culture and community, degradation of chlorinated aromatics can proceed along different pathways (van der Meer et al., 1992). The aerobic degradation of natural aromatic compounds usually proceed via their activation by oxygenases and a few central dihydroxylated intermediates such as catechol, chlorocatechol and gentisate, which after dioxyphenolytic ring cleavage are finally channelled into the TCA cycle. As an example phenol, is usually subject to monooxygenation to catechol followed by extradiol cleavage. Benzoate is also usually activated to catechol, degradation however follows an intradiol cleavage route, the 3-oxoadipate pathway. Whereas numerous enzymes capable of activating aromatic compounds such as benzoate dioxygenase are of broad substrate specificity and capable to transform halogenated substrate analogues, neither the catechol *meta*-cleavage pathway nor the 3-oxoadipate pathway are suited for channelling chlorocatechols in the tricarboxylic acids cycle (TCA) cycle. Bacteria capable to degrade chloroaromatics via chlorocatechols usually contain a chlorocatechol *ortho*-cleavage pathway. This pathway comprises a chlorocatechol 1,2-dioxygenase, which differs from catechol 1,2-dioxygenases of the 3-oxoadipate pathway by its broad substrate specificity and capability to cleave 3- and 4-chlorocatechol (4-CC) and various dichlorocatechols with high activity (Dorn & Knackmuss, 1978a; Reineke, 1999). The formed chlorosubstituted muconates are further transformed by chloromuconate cycloisomerase. Proteobacterial chloromuconate cycloisomerases differ from muconate cycloisomerases in various aspects. First, chloromuconate cycloisomerases catalyse a cycloisomerisation and dechlorination of 3-chloromuconate to give *cis*-dienelactone, whereas muconate cycloisomerases catalyse an additional decarboxylation with protoanemonin as product. The consortium members of the 4-CS degrading consortium inherit these different enzymes. *P.* sp. MT 1 is able to degrade 4-CC with the catechol 1,2-dioxygenase and the 3-oxoadipate way, whereas *A. xylosoxidans* MT 3 contains the enzymes of the chlorocatechol pathway.

1.4.5 Network of 4-chlorosalicylate degradation

The carbon sharing in this 4-CS degrading community was previously described by Pelz et al. (1999a; 1999b). The degradative pathway shown in Figure 1 has been proposed. The authors found that *P.* sp. MT 1 is the only member able to metabolise 4-CS, which is subject to monooxygenation by the enzyme salicylate 1-hydroxylase. In *P.* sp. MT 1 degradation of 4-CC follows intradiol cleavage and the 3-oxoadipate way. The intradiol cleavage product is 3-chloro-*cis,cis*-muconate, which is transformed to an enolate, which is highly unstable. The enzyme muconate cycloisomerase transforms the enolate to 4-chloromuconolactone. Another reaction product is *cis*-dienelactone, which originates at a rate of 10%. A probably

spontaneous decarboxylation reaction of 4-chloromuconolactone generates the product protoanemonin (Blasco et al., 1995). In *P.* sp. MT 1, the chloride of 4-chloromuconolactone is substituted by a hydroxyl group, this reaction is catalysed by the enzyme *trans*-dienelactone hydrolase. The resulting 4-hydroxymuconolactone is the same as maleylacetate and can be transformed to 3-oxoadipate. This is a substrate for the tricarboxylic acids cycle. The hypothetical 4-CS degradation pathway by *P.* sp. MT 1 was recently revealed by Nikodem et al. (2003). However, if maintained alone at high growth rates, metabolites, which spill out from this pathway would toxify *P.* sp. MT 1.

The first metabolite, 4-CC, leaks out at a rate of about 10% of the substrate carbon from *P.* sp. MT 1 and 4-CC is very efficiently taken up by *A. xylosoxidans* MT 3. *A. xylosoxidans* MT 3 thereby protects the consortium from the toxicity that would otherwise originate from accumulation of 4-CC. In *A. xylosoxidans* MT 3 4-CC is further degraded by the chlorocatechol pathway. The enzyme chlorochatechol 1,2-dioxygenase, which enables the strain to specifically transform chlorinated catechol in this uncomplicated way to a TCA-cycle member, comprises the specific advantage of *A. xylosoxidans* MT 3, which therefore plays a significant role in the consortium. The cleavage product is - as in *P.* sp. MT 1 - 3-chloro-*cis,cis*-muconate,which is transformed to an enolate. The further degradation of this enolate by *A. xylosoxidans* MT 3 differs from the 3-oxoadipate way of *P.* sp. MT 1. The enzyme chloro-muconate cycloisomerase catalyses a cyclysation and dechlorination step, which is not followed by a decarboxylation. This reaction transforms all enolate to *cis*-dienelactone, no 4-chloromuconolactone is produced. *Cis*-dienelactone is transformed to maleylacetate by a hydrolytical ring-cleavage reaction. The enzyme maleylacetate reductase reduces this substance with the help of $NADH_2$, to 3-oxoadipate, which can be transformed to a member of the TCA (Kaschabek & Reineke, 1995). Between the pathway of *P.* sp. MT 1 and the pathway of *A. xylosoxidans* MT 3 are three major differences: 1. The enzyme chlorocatechol 1,2-dioxygenase cleaves 4-CC with high activity. 2. The enzyme chloromuconate cycloisomerase transforms the enolate to *cis*-dienelactone/protoanemonin at a ratio of 100/1, wheras the ratio of muconate cycloisomerase is *cis*-dienelactone/protoanemonin 1/10. 3. The produced metabolites of the two pathways are similar, but in *A. xylosoxidans* MT 3 *cis*-dienelactone is directly transformed to maleylacetate, whereas in *P.* sp. MT 1 the enolate is transformed to 4-chloromuconolactone, an additional metabolite of the *P.* sp. MT 1 pathway.

The second important carbon spill by *P.* sp. MT 1 is protoanemonin, which has broad antibiotic activity (Didry et al., 1991). C*is*-dienelactone and protoanemonin are dead-end products for *P.* sp. MT 1. Protoanemonin has been found to be formed during microbial metabolism of chlorinated aromatics as toxic dead-end product (Blasco et al., 1995).

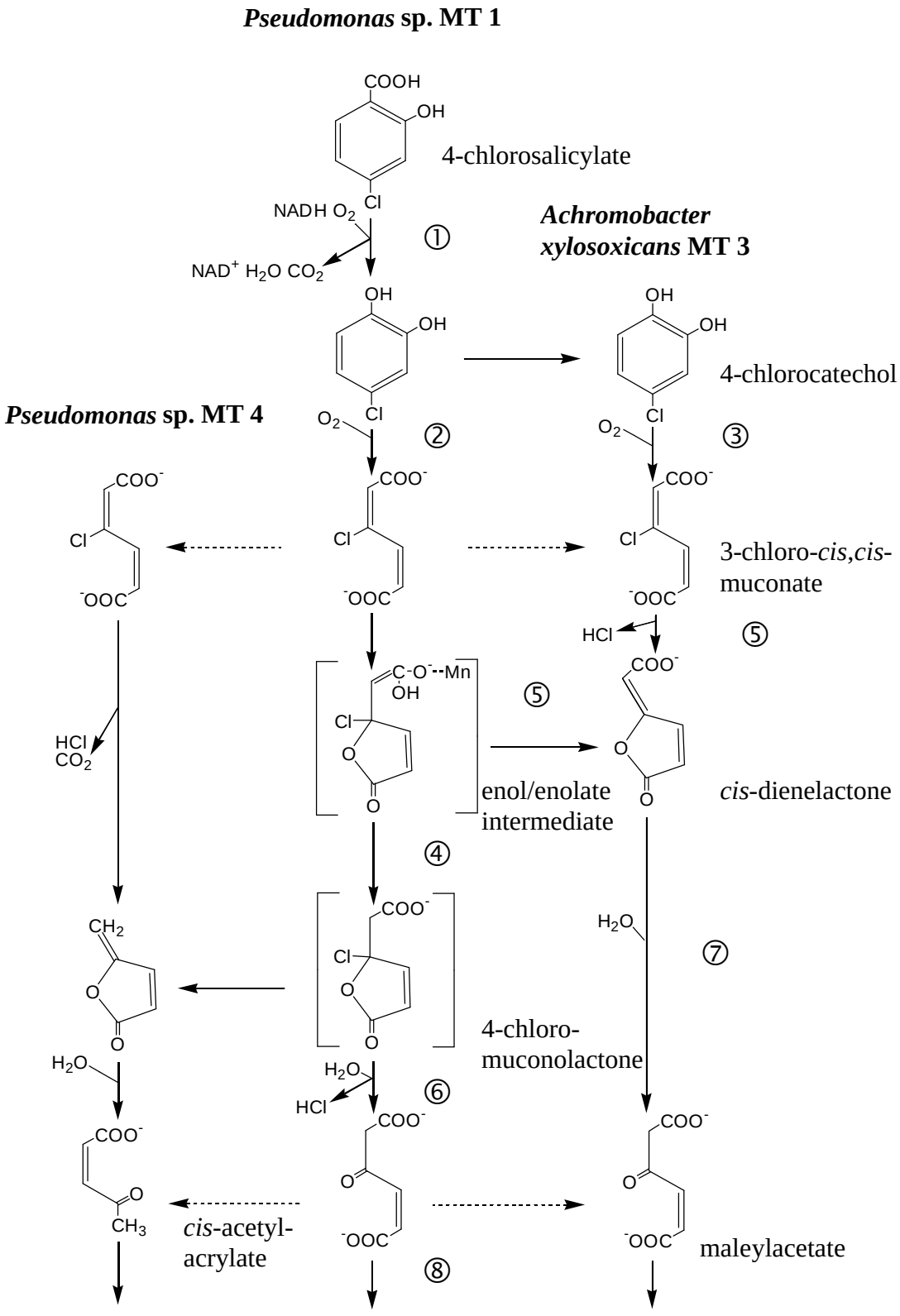

Figure 1: Carbon flow in the 4-CS degrading consortium. Presumably involved enzymes: ① salicylate hydroxylase, ② catechol 1,2-hydroxylase, ③ chlorocatechol 1,2-hydroxylase, ④ muconate cycloisomerase, ⑤ chloromuconate cycloisomerase, ⑥ *trans*-dienelactone hydrolase, ⑦ *cis*/*trans* dienelactone hydrolase, ⑧ maleylacetate reductase

The formation of protoanemonin represents a severe danger of poisoning for all members of the microbial consortium (Blasco et al., 1997). *P.* sp. MT 4 takes up and metabolises protoanemonin efficiently and thereby protects *P.* sp. MT 1 from suicide poisoning.
Probably in *P.* sp. MT 4 a novel pathway operates with toxic protoanemonin as a critical metabolite that is channelled via *cis*-acetylacrylate by as yet unknown reactions, most probably involving the intermediate levulinic acid, into the TCA. In a different strain, *Pseudomonas* sp. B13, protoanemonin was transformed by the dienelactone hydrolase to *cis*-acetylacrylate (Brückmann et al., 1998).

The metabolic roles of the single members of the consortium are reflected in their abundances. The primary degrader *P.* sp. MT 1 is the most abundant (80%), the secondary metabolisers, *A. xylosoxidans* (16%) and *P.* sp. MT 4 (3%), are less abundant. The role of MT 2 (*Empedobacter brevis* /*Flavobacterium breve*), the fourth consortium member, which is only 1% abundant, is unknown.
The characteristic parameters of the 4-CS degrading consortium, such as growth rates and yields, have to be acquired as a basic prerequisite for the control of the undisturbed consortium and to study the influence of disturbances.

1.5 Aim of the work

The aim of this thesis was to determine how the 4-CS degradation and the structure of a model consortium was affected by typical natural ecological perturbations and the consortium's responses to such perturbations. A 4-CS degrading consortium, consisting of four microbial strains, which was originally derived from the sediment of a polluted small stream, served as a model. The consortium was maintained in continuous culture and subjected to changing physicochemical and biotic factors. The great advantage of the model system is, that influences upon structure and function of the consortium can be monitored simultaneously, which will allow a deeper insight in the stress tolerance reaction mechanisms of the consortium.

The identification of influential parameters and the study of response mechanisms will support the application and enhance the usefulness of microbial consortia in bioremediation and other related fields. Not only the detection of critical ecological parameters, which regulate and influence microbial communities, but also the knowledge of response mechanisms will aid many practical applications of microbial consortia. Applications are numerous situations in which a process occurs at the level of a consortium, community or microenvironment, including bioremediation, industrial and municipal wastewater treatment, composting, biological control agents biofertilisers and oral, skin or intestine biofilms in humans.

1.6 Leading questions:

- What are the characteristic activity determining parameters (consortium composition, growth rate, degradation rate) of the 4-CS degrading consortium and strain *P*. sp. MT 1 in batch culture?
- How do the following disturbances influence structure and function of the consortium growing in continuous culture and degrading 4-CS, and what are the responses:
    - addition of second carbon sources;
    - addition of competing strains (environmental isolate and GEMs);
    - reduction of the oxygen concentration.

1.7 Tools to study microbial community structure and function

Among the wealth of methods available to monitor the structure of microbial communities, a microscopical technique combined with immunological methods was applied to monitor the abundance of the consortium members. High-performance liquid chromatography (HPLC) was applied to monitor the 4-CS degradation. The following chapter explains the selection of these methods.

Numerous procedures are available for monitoring changes in community structure (e.g., culture-based analyses, community level physiological profiling (Engelen et al., 1998; Konopka et al., 1998), analysis of the lipid content of microbial cells (e.g., phospholipid fatty acid analysis (Bundy et al., 2002; Ringelberg et al., 2001)), and molecular genetic techniques (ref. see below); theses approaches each have biases and limitations ((Busse et al., 1996; Garland, 1997; Ogram & Feng, 1997; Stahl, 1997; White et al., 1997)).

Several nucleic-acid based methods (Amann et al., 1995; Hurst, 1997), such as retrieving rRNA genes (mostly 16S ribosomal DNA (rDNA) and 18S rDNA) from the environment and comparison to large databases, are labour intensive and time-consuming. In general, almost all methods which are based on the multiplication of DNA-pieces by polymerase chain reaction (PCR) contain various biases (Wintzingerode et al., 1997).

Among the microscopy based techniques, both fluorescence in situ hybridisation (FISH) and labelling with fluorochrome-conjugated antibodies are useful. Fluorescein-labeled oligonucleotide probes specific for the highly conserved multicopy 16S ribosomal RNA subunit (16S rRNA) can be used to monitor bacterial growth in mixed microbial populations (Amann et al., 1990; Troussellier et al., 1993). However, they can not as yet be used non-destructively as in the case of fluorescent antibodies. Another difficulty in applying FISH is that in environmental samples, only low numbers of the target molecule rRNA exist in the cells, and therefore the intensity of the staining is often not sufficient for detection.

1.7.1 Immunological approach

Due to the above mentioned reasons, the structure of the consortium and the abundance of the single consortium members was assessed by epifluorescence enumeration of cells labelled by indirect immunofluorescence (IIF). An advantage of this method is that the microscopic analysis allows one to achieve a picture of the cell form and therefore indicates the physiological state of the cells; for example "ghosts", cells which exhibit ring fluorescense but no DNA staining, indicate dead cells. The general drawback of the application of fluorochrome-conjugated antibodies is the generation of the antibodies. Pure cultures of the four consortium members were available; and specific antibodies had been previously generated (Faude, 1996).

The potential of immunochemical test systems is useful in the investigation of environmental samples (McFeters et al., 1995). This method has been successfully used for the identification of metabolic groups of bacteria (Assmus et al., 1997; Zellner et al., 1995), to distinguish between the members of metabolic groups (de Macario et al., 1982), for analysis of microbial ecosystems (Hahn & Höfle, 1998) and for tracking genetically engineered (Brettar et al., 1994; Ramos-González et al., 1992) or indigenous bacteria (Faude & Höfle, 1997; Fuller et al., 2001).

Advances in immunochemical detection of microorganisms were reviewed by Hock (1996). Fluorescence microscopic techniques are sensitive due to the huge contrast between the labelled areas and the non-illuminated background. The main advantage is the identification of single cells and the quantification with high precision and detection level, if the method is properly applied, e.g. the same person performs the counting and the number of counted cells is between 200-500 cells per filter.

1.7.2 Analytical chemistry

The abundance of test chemicals in experimental vessels are in general monitored by direct procedures which utilise specific chemical purification and/or detection instruments (including gas chromatography, gas chromatography-mass spectrometry, HPLC, spectrophotometry and radioisotopic tracers). The concentration of the main substrate 4-CS was measured by HPLC analysis and the rate of decrease, which represents the metabolic activity of the consortium, was calculated. Additionally the occurrence of metabolites (e.g., 4-CC, protoanemonin) can be monitored (Blasco et al., 1995). In this work HPLC was selected, as this is the traditional investigation technique (in combination with enzyme assays), which is usually applied in biodegradation studies (Prucha et al., 1997).

For the concentration assessment of additional applied carbon sources, dissolved organic carbon (DOC) was measured as a sum parameter. The concentration of one carbon source, 4-CS could be estimated by HPLC measurement and the difference to the total sum of DOC displays the concentration of the second carbon source, if only two carbon sources were applied and no metabolites were present.

2 Materials and Methods

2.1 Appliance

Autoclave	Tecnoclav 50, Tecnomara AG, Zürich, Schweiz
CCD Camera	INTRAS, Infors AG
Software	Image Pro II
Climatic chamber	Type 3500; RUMED, Rubarth Apparate, Germany
Cooling centrifuge	RC3C, RC5C Sorval Instruments, Du Pont, USA
DOC	DIMATOC 100, DIMATEC
Elisa reader	Precision microplate reader, Molecular devices
Epifluorescence microscope	Photomicroscope Auxiophot, Carl Zeiss, Germany
French press	Aminco, American Instruments Comp., Silver Spring, USA
HPLC	Waters 712 WISP high pressure liquid chromatograph
Magnetic stirrer (1 chemostat)	Ikamak RCT basic, IKA Labortechnik
Magnetic stirrer (6 chemostats)	Multiport, H & P Labortechnik
Oxygen electrode	Inpro 6000, Mettler-Toledo, Germany
Enhancer	Type 170% Air, Mettler-Toledo, Germany
Recorder	Logoscreen, paperless recorder, Dataloger with graphical display, M. K. Juchheim, Germany
Software	Jumo-PCA, M. K. Juchheim
Peristaltic pump	Watson Marlow, England
pH meter	761, Calimatic
Photospectrometer (enzyme analysis)	UV 2100 Shimadzu Corp., Japan
Photospectrometer (OD_{600})	Spectronic 601, Milton Roy
Precision balance	AE 260, Mettler
Rotary shaker (5 ml vials)	Gyrotory shaker, New Brunswick Scientific Corp., USA
Rotary shaker (flasks)	Pilot-shake RC-6-U, System Kühner
Table shaker	Infors AG, Bottmingen
Thermomixer	Thermostat 5436, Eppendorf,
Ultra sonicator	Ultraschall Dislocator Labsonic U, Braun, USA
Ultra-centrifuge	TL-100, Beckmann Instruments Inc., USA
Vortex	Genie 2™ Bender + Holbein, Zürich, Switzerland;

2.2 Chemicals

All Chemicals (except 4-chlorosalicylate (4-CS), which comprised 93% purity, Aldrich Germany (Pelz, 1999a)) had p.A. purity and were supplied from Boehringer Mannheim, Difco, Merck, Oxoid, Riedel de Haen, Roth, Serva or Sigma. Fluorescent dyes and conjugates were supplied from Boehringer Mannheim, Beckton Dickinson, Dianova, Hoechst, Polysciences and Sigma.

2.3 Media

The media were prepared with deionised water. Solid plates were prepared by addition of 15 g agar l^{-1} medium prior to autoclaving.

2.3.1 **4-chlorosalicylate medium**

Part 1:	$Na_2HPO_4 \times 2\ H_2O$	78	g
	KH_2PO_4	68	g
	in H_2O	1000	ml
Part 2:	$MgSO_4 \times 7\ H_2O$	4.1	g
	$Ca(NO_3)_2 \times 4\ H_2O$	0.5	g
	$NaNO_3$	0.85	g
	Fe(NH_4)-citrate	0.1	g
	in H_2O	990	ml
	ad trace element solution	10	ml
Part 3:	4-chlorosalicylate	8.63	g
	10 N NaOH	7.5	ml
	in H_2O	300	ml
Trace element solution:	HCl (25%)	1.3	ml
	$ZnCl_2$	0.07	g
	$MnCl_2 \times 4\ H_2O$	0.1	g
	H_3BO_4	0.062	g
	$CoCl_2 \times 6\ H_2O$	0.19	g
	$CuCl_2 \times 2\ H_2O$	0.017	g
	$NiCl_2 \times 6\ H_2O$	0.024	g
	$NaMoO_4 \times 2\ H_2O$	0.036	g
	in H_2O	1000	ml

7700 ml Milli Q water were autoclaved for 20 min at 121 °C in a 10 l glass vessel (Schott, Germany). Each part of the medium was prepared separately, filter sterilised (pore size 0.2 µm, Nalgene) and added to the cooled water in the feedstock vessel. The pH of the medium was 6.8.

2.3.2 Other carbon sources

For liquid (and solid) cultures with different carbon sources variations of the above described 4-chlorosalicylate medium were used. Part 3 was substituted by an aqueous solution of the carbon source of interest. In this work the following carbon sources were used and applied to a final concentration of:

4-chlorocatechol[a]	5 mM
histidine	5 mM, 6 mM
ethanol[b]	5 mM, 6 mM
iso-propanol[b]	5 mM, 6 mM

[a] this carbon source was prepared as 10 × stock, the medium was prepared each day and connected to the feed, because the catechols polymerised and could then not be metabolised by the bacteria anymore; [b] the alcohols were added directly, the missing volume in the medium was substituted with water

2.3.3 Nutrient broth (NB) medium

Beef extract	3	g
Peptone	5	g
in H_2O	1	l

The NB was usually $^{1}/_{10}$ diluted, to reach a similar concentration of dissolved organic carbon as 5 mM 4-CS.

2.4 Bacterial strains

2.4.1 Chemostat isolates

The 4-CS degrading consortium was previously generated by continuous culture of a sediment sample. The taxonomic position of the 4 predominant isolates was determined by low molecular weight RNA profiles, BIOLOG™ and 16S rDNA gene sequencing, the strains were identified as *Pseudomonas* sp. (MT 1), *Empedobacter brevis* (named previously *Flavobacterium breve*) (MT 2), *Achromobacter xylosoxidans* (named previously *Alcaligenes xylosoxidans*) (MT 3) and *Pseudomonas* sp. (closely affiliated to *Pseudomonas fluorescens*) (MT 4) (Faude, 1996).

2.4.2 Competitor and reference strains

The consortium, which was continuously cultured in a chemostat, was subjected to disturbances. Biotic stress was the introduction of a competing bacterial strain, which has similar or superior degrading abilities as strains of the consortium. One additional bacterial strain was used as positive control in enzyme tests. The strains are listed in Table 2.

Table 2: Names, taxonomy (assessed by 16S rDNA sequencing), physiological properties and references of bacterial strains used in this thesis

name	genera, family	properties[a]	source	reference
B13 SN45P	*Pseudomonas* sp.	chlorocatechol *ortho*-cleavage pathway; Km^R	Dietmar Pieper	(Müller et al., 1996) (Erb et al., 1997) (Eichner et al., 1999)
G7::4/4	*Pseudomonas putida*	Nah^+ chlorocatechol *ortho*-cleavage pathway	Rolf Wittich	(Morris & Barnsley, 1982) (Jakobs, 1997) (Dunn & Gunsalus, 1973)
RW10	*Pseuomonas putida*	salicylate 1-hydroxylase, catechol *ortho*-cleavage pathway	Rolf Wittich	(Wittich et al., 1999) (Blasco et al., 1995)
A02	*Pseuomonas putida*	salicylate 1-hydroxylase	Dietmar Pieper	no reference environmental isolate from Elbe river

[a]abbreviations: Km^R = Kanamycin resistance; Nah^+ = The plasmid NAH7 encodes for genes responsible for the 14-step pathway for degradation of naphthalene to pyruvate and acetaldehyde through the intermediate salicylate;

2.5 Culture conditions

2.5.1 Stock-culture

The stock cultures of the single strains were stored on NB plates at 4 °C. The cultures were transferred to new plates every 4-6 weeks or prior to an experiment. In addition the pure cultures were stored in glycerol. To 1 ml liquid culture 500 µl sterile glycerol (87%) was added and the mixture vortexed. The samples were stored in storage tubes with screw caps at -70 °C. The cultures were cultivated in liquid medium all 6-12 month and new glycerol cultures were prepared.

2.5.2 Solid culture

Cultures on solid media were used for purity control and as inoculum for batch experiments. An aliquot of 100 µl of liquid culture or diluted sample were spread on the plates using a Drigalski spatula. Or a sample of the glycerol storage was streaked by means of an inoculation needle. The plates were incubated at 30 °C 1-3 days. NB plates or selective plates were used.

2.5.3 Liquid (batch-) culture

Liquid cultures were used for biomass production, to assess the growth parameters of *P*. sp. MT 1, of the mixed culture and of the competitors and to observe substrate utilisation abilities of the single strains. The inoculum of consortium cells was derived from a continuous culture. Pure culture inoculum was one colony from a NB plate, on which the glycerols were streaked.

2.5.3.1 Liquid culture for the production of antigens and biomass

For the production of biomass for the preparation of antigens, sterilised glass tubes containing 5 ml NB medium were inoculated with single colonies from plates. They were grown on a rotary shaker at 186 rpm at 30 °C over night, until they reached an OD_{600} of 0.3.

2.5.3.2 Liquid culture for *P.* sp. MT 1, consortium and competitor growth parameter measurement and for mixed substrate experiments

Baffled Erlenmeyer flasks were filled 20% of the flask volume with liquid medium and inoculated with either 1-10% of the liquid volume with liquid culture or with cultures from plates. For the growth parameter experiments 4-CS medium with varying 4-CS concentrations were used, for the mixed substrate experiments the second substrate was applied to the medium previous to inoculation.

2.5.3.3 Liquid culture to test substrate utilisation

To test the substrate utilisation of the single strains of the consortium, the pure cultures were cultivated in 96-well microtiterplates. 270 µl of media containing different carbon sources were filled in the wells and 30 µl of pure culture (pregrown in batch culture) OD_{600} = 0.2, were added, this resulted in a start OD_{600} = 0.04, as the OD_{600} of the medium was = 0.02. The change in the optical density was monitored at 600 nm in an Elisa reader. Negative controls for contamination (media without cells) and positive controls for fitness of the culture (nutrient broth) were included.

2.5.3.4 Treatment of *Pseudomonas* strains for the use in competition and enzymatic analysis experiments

The *Pseudomonas* strains were pregrown in 200 ml baffled Erlenmeyer flasks with 25 ml 4-CS or NB medium, inoculated with colonies from NB plates, then transferred to 2.5 l baffled Erlenmeyer flasks with 500 ml 4-CS or NB medium, both incubated at 30 °C on a rotary shaker. The cultures were washed by centrifugation at 12000 *g* (7000 rpm) for 10 min and resuspended in 50 mM $MgSO_4$. The cells for the enzymatic analysis were further treated

as described later (2.7.1). For the competition experiments some batch cultures were combined, depending on the optical density. After a second centrifugation the cultures were suspended in 50 ml 50 mM $MgSO_4$. The optical density was adjusted to 10 × the optical density of the chemostat culture, thus the optical density of the competitor and the consortium were the same after addition of the competitor.

2.5.4 Fed-batch culture

Fed batch cultures were used to acquire data in a limited time course (pre-experiments). They were used to get preliminary results about the mixed substrate utilisation experiments. Up to 10 250 ml baffled Erlenmeyer flasks were inoculated with 50 ml consortium inoculum and placed on a table shaker and maintained under the conditions listed in Table 3. Medium was fed by a peristaltic pump for two weeks. The reaction vessel and the medium supply were supplied with sterile air filters to ensure sterile air influx.

Table 3: Maintenance conditions for fed-batch culture

conditions	**fed-batch culture**	
size of reaction vessel	250	ml
volume (at beginning)	50	ml
volume (at end)	200	ml
flow rate	0.5	ml h^{-1}
shaking rate	110	rpm
temperature	22	°C
pH	6.8	

2.5.5 Chemostat culture

In the present work, the chemostat was a carbon limited culture system. The 4-CS degrading consortium (defined mixed culture) was cultivated until a steady state was reached, then the influences of changing factors (mixed substrate utilisation, competing strains and others) on the composition and function of the community were studied.

2.5.5.1 Construction

The construction of the reactor is shown in Figure 2. The reactor consisted of a glass culture vessel, in which the organisms were grown. Sterile growth medium was fed into the culture vessel (at a steady flow rate), from the 10 l storage bottle driven by a peristaltic pump. The medium was lead through a drip trap, then directly dripped into the culture by means of a hypodermic needle through a silicone membrane inserted in the screw-cap of the flask. Culture emerged from the culture vessel at the same rate, through a constant level device. The efflux was led through another drip trap in a 10 l waste bottle. Air was supplied by an in house press air connection, the flux was measured by means of a flow meter (FP-1/4-10-G-5,

Bailey-Fischer & Porter). The air stream was lead through sterile air filters (Midisart 2000, Sartorius) to the bottom of the culture bottle and into the two drip traps. The contents of the reaction vessel were stirred by a Teflon-coated stirring bar, to disperse the growth medium instantaneously and uniformly throughout the vessel.

All the material used (glassware, connections, silicon-tubes) were autoclaved prior to use. For the connections (for example between the medium supply and the reaction vessel) were screw threat adapter couplings and glass screw thread adapters, size GL 18, used, which were flamed for sterilisation.

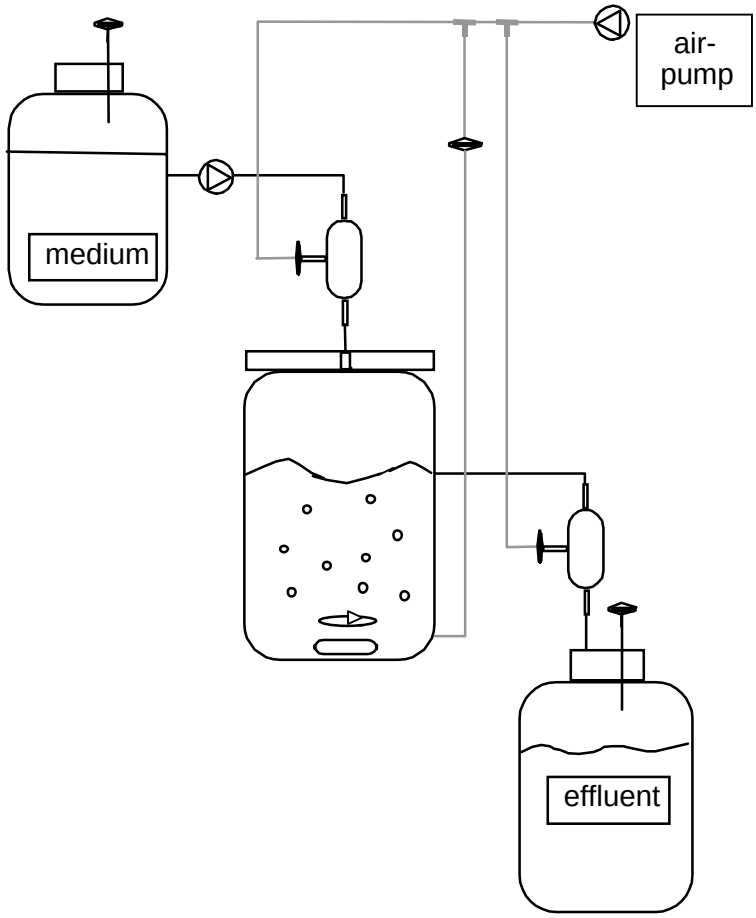

Figure 2: Construction of the reactor for the continuous culture of the consortium. Grey lines indicate oxygen flux, black lines indicate medium flux.

2.5.5.2 Pre-culture

To start a new mixed culture the four strains were enriched separately to avoid contamination. 25 ml liquid NB medium in 100 ml baffled Erlenmeyer flasks were inoculated with colonies of the four pure strains picked from NB plates (see 2.5.3.1). After 2-3 days of incubation at 120 rpm on a large shaking machine at 30 °C, 250 ml of NB medium in 1 l baffled Erlenmeyer flasks were inoculated with 5 ml culture of the 100 ml flasks. Before each transfer step the purity of the cultures were tested by plating on NB plates and control for inhomogen colony morphology and by indirect immunofluorescence (IIF) of the cultures (with the specific antibodies). After another 2-3 days the pure cultures were washed with sterile 50 mM

$MgSO_4$ solution (centrifugation at 12000 *g* (7000 rpm) for 10 min), resuspended in 50 ml 50 mM $MgSO_4$ solution and combined. The mixed culture served as inoculum for one 2.5 l baffled Erlenmeyer flask with 500 ml 1 mM 4-CS medium. After 5 days incubation at 30 °C, 50 ml of this mixed culture were used to inoculate six 2.5 l baffled Erlenmeyer flasks with 500 ml 1 mM 4-CS medium. After another five days these cultures were inserted in the 500 ml reaction volume chemostats and the feeding with 5 mM 4-CS was started with a low dilution rate ($D=0.02\ d^{-1}$).

2.5.5.3 Pre-steady-state conditions

The dilution rate at the start of the continuous culture was $D = 0.02\ d^{-1}$, this rate was stepwise increased until it finally reached $D = 0.1\ d^{-1}$. After each addition, the dilution rate should be left unchanged until the new steady state was reached (3 times the volume exchange ($\tau = 3$)), but this would take a long time (e.g. for D = 0.02 129 days). However, in the case that after 2 - 3 days no 4-CS accumulation was observed, was the dilution rate increased further. This dilution rate increase resulted in an increase in cell number, which can in some experiments be seen at the early stage of the continuous culture. Generally, at a Dilution rate of $D= 0.1\ d^{-1}$, a steady state would be reached (for $\tau = 3$) after 30 days. However, if the optical density and cell number did not vary much after 7 days, and if the consortium composition did meet the criteria for undisturbed growth, the disturbance was applied earlier.

2.5.5.4 Six 500 ml reactors

The consortium was maintained parallel in 6 chemostats with a reaction volume of 500 ml. All chemostats were performed and observed in the same way, to ensure the same steady state conditions. Maintenance conditions are summarised in Table 4.

2.5.5.5 Twin 2500 ml reactors for oxygen-limiting experiment

To study the influence of the oxygen reduction on the consortium, two 2500 ml reaction volume chemostats were used. The chemostats were filled with 500 ml consortium culture, then medium was fed with a low dilution rate ($D = 0.01\ d^{-1}$) until the reaction volume of 2500 ml was reached. Then the dilution rate was stepwise increased ($D = 0.02, 0.05, 0.07\ d^{-1}$) until the dilution rate of $0.1\ d^{-1}$ was reached. Maintenance conditions are summarised in Table 4. The oxygen concentration was measured by means of oxygen electrodes.

Table 4: Maintenance conditions of six 500 ml reactors and twin 2500 ml reactors

conditions	**500 ml reactors**		**2500 ml reactors**	
size of reaction vessel	1000	ml	5000	ml
working volume	500	ml	2500	ml
flow rate	50	ml d^{-1}	250	ml d^{-1}
dilution rate	0.1	d^{-1}	0.1	d^{-1}
air supply	1	vvm	1	vvm, later reduced in one reactor
stirring rate	150	rpm	400	rpm
temperature	20 ± 2	°C	12	°C
pH	6.8		6.8	

2.5.5.6 Assessment $k_L a$ value

The influence of reduced oxygen concentration on structure and function of the 4-CS degrading consortium was studied. A value to describe the efficiency of the reactor system to transport oxygen into the reaction liquid is the $k_L a$ value (oxygen mass-transfer coefficient for the mass-transfer area a). The dynamic method was applied to measure and calculate the $k_L a$ value of the 2.5 l reactor filled with minimal medium, but without cells. Oxygen and nitrogen were supplied by an in-house line, through a flow meter. The oxygen was measured with O_2-electrodes, enhancer and a recorder. The development of the oxygen concentration is shown in Figure 3A. The broth was de-oxygenated by sparging nitrogen into the culture vessel. Dissolved-oxygen concentration dropped during this period. Air was then pumped into the broth at a constant flow-rate and the increase in dissolved-oxygen concentration was monitored as a function of time. During the re-oxygenation step, the system was not at a steady state. The rate of change in dissolved-oxygen concentration during this period is equal to the rate of oxygen transfer from gas to liquid. The increase in C_{AL} was monitored as a function of time with a paperless recorder. The analysis was done by integration of the measured curve. $k_L a$ was estimated using the equation:

$$k_L a = \frac{\ln\left(\frac{\overline{C}_{AL} - C_{AL1}}{\overline{C}_{AL} - C_{AL2}}\right)}{t_2 - t_1}$$

$k_L a$ is the combined mass-transfer coefficient in units of s^{-1}, $\overline{C}_{AL}$ is the final steady state dissolved-oxygen concentration, C_{AL1} and C_{AL2} are two oxygen concentrations measured during re-oxygenation at times t_1 and t_2, respectively.

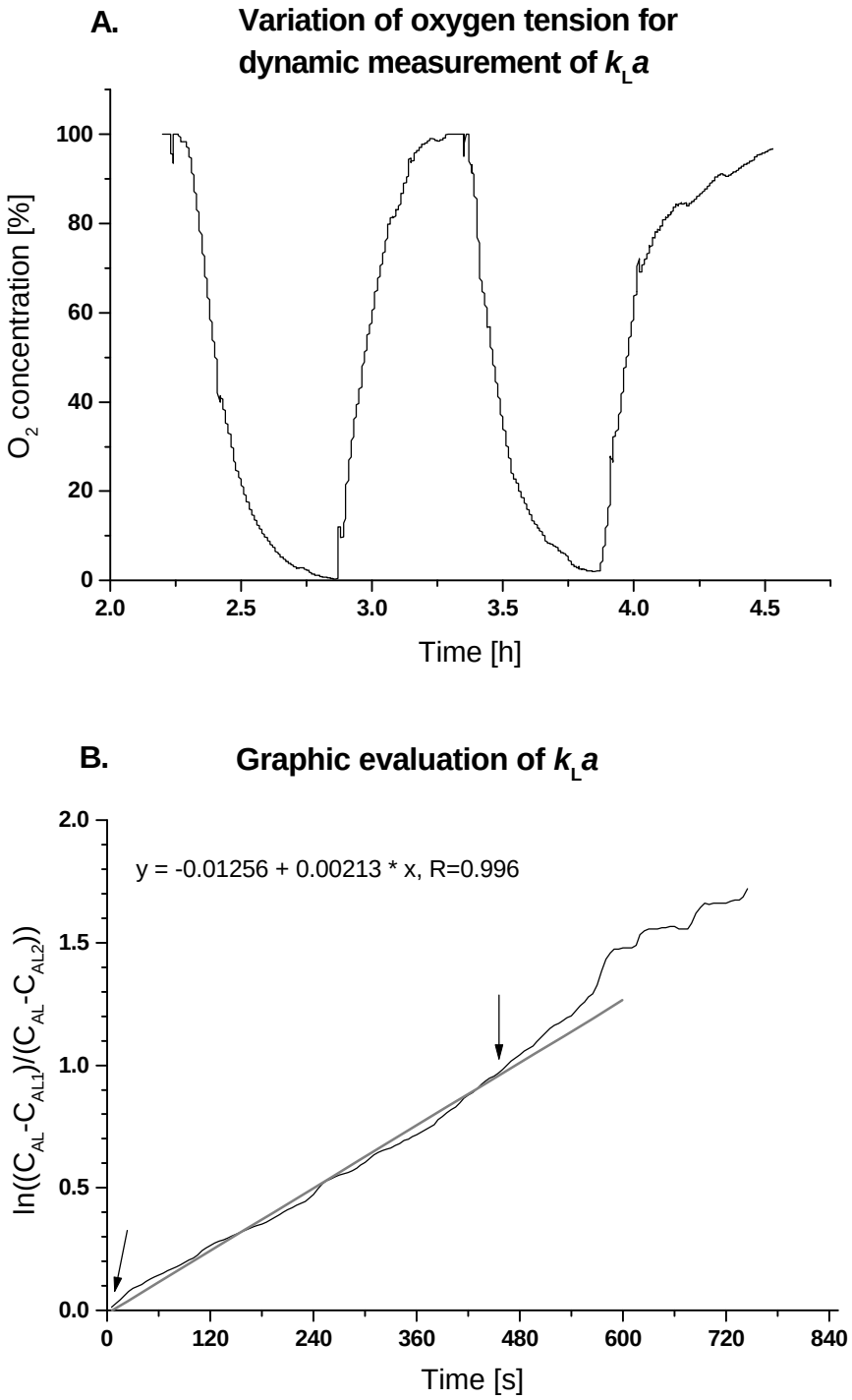

Figure 3: A. Variation of oxygen tension for dynamic measurement of k_La, nitrogen and oxygen sparked by air influx; B. Graphic evaluation of k_La of fermenter without cells, air influx by air inlet; analysed data of 2.9 h to 3.15 h of graph A., linear fit of selected data (between arrows) (grey line) revealed equation shown above, R = Regression coefficient

The k_La value was determined for the time range of 2.9 h to 3.15 h (Figure 3B) and for the time range between 3.85 to 3.95 h (data not shown) in Figure 3A. The oxygen was supplied over the gas influx at the bottom of the reactor with 1 vvm (2.5 l reaction volume and 2.5 l/min air influx). The k_La was found to be 2.13 - 2.16 × 10^{-3} s^{-1}.

2.5.5.7 Reduction of oxygen concentration by control of inflowing gas amount

Generally was the oxygen supplied via air influx over gas-flow meters. As a disturbance was the air influx in one reaction vessel reduced to the desired level with the beforehand used flow meter (FP-1/4-10-G-5, Bailey-Fischer & Porter), but to obtain a lower flux, a smaller flow meter (FP-1/16-12-G-5, Bailey-Fischer & Porter) was used. The flux was 1 vvm (volume/(volume min^{-1})) in the undisturbed control. In the disturbed reactor the flux was reduced to 0.1, 0.01 and finally to 0.006 vvm.

2.5.5.8 Sampling of cells and medium

The sampling device was a glass screw thread tube, closed with plastic screw caps, size GL 18, connected on a second device to a sterile filter with 0.2 µm pore size (Minisart RC, Sartorius) with a syringe. The glass tube was flamed before the sample was taken, afterwards the culture broth in the silicone tubing was pressed back into the culture vessel with sterile air, by means of the syringe.

20 ml sample were taken three times a week out of the continuous culture and processed as described here. In the batch and fed-batch experiments only 2 ml samples were taken and smaller aliquots (in brackets) stored.

- optical density: 1 ml (0.5 ml) sample was transferred to a standard cell (Halfmicrocuvette, Greiner);
- HPLC and DOC analysis: 4 ml (1 ml) sample were filter sterilised with 0.2 µm pore size, (Minisart RC, Sartorius) and stored at -20 °C.
- IIF and cell number counting: 2 ml (0.5 ml) sample were supplied with 120 µl (30 µl) 36.5% formalin solution and stored at 4 °C;

Stability of the pH and contamination control was monitored at irregular intervals:

- Colony forming units: 10-fold serial dilutions in sterile phosphate buffered saline (PBS) were made. 100 µl of the 10^{-5} and 10^{-6} diluted sample were spread on NB plates.
- pH: 2 ml sample were filter sterilised and measured with a pH meter.

Phosphate buffered saline (PBS)	NaCl	8	g
	KCl	0.2	g
	Na_2HPO_4	1.44	g
	KH_2PO_4	0.24	g
	adjust pH with HCl to	7.4	
	in H_2O	1000	ml

2.6 Analysis of bacterial biomass and bacterial cell counts

2.6.1 Biomass (dry weight)

For the analysis of some experiments it was necessary to translate the culture turbidity into the biomass. For the correlation between optical density and biomass of the consortium a calibration curve was generated. Dilutions of the culture broth (from continuous culture) to the optical densities in the observed ranges were prepared in triplicates, the highest optical density was prepared in duplicates. 250 ml culture broth were centrifuged at 12000 *g* for 15 min. Then the supernatant was discarded and the pellet resuspended in 9 ml PBS or 50 mM $MgSO_4$. The concentrated suspension was transferred to 50 ml centrifuge tubes (Corex). These were previously dried at 105 °C in a drying oven, cooled in an exsikator and weighted on a analytical balance. The concentrated culture was centrifuged again for 15 min at 12000 *g*. When the supernatant was discarded, the pellet was dried at 105 °C in the centrifuge tube until weight constancy.

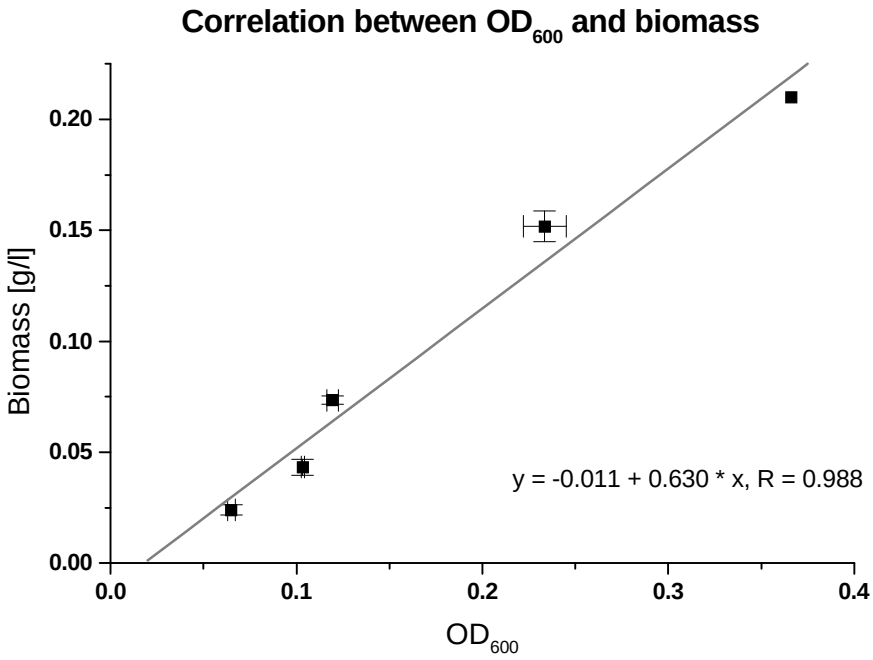

Figure 4: Correlation between OD_{600} and biomass (■) of the 4-CS degrading consortium. Linear fit of data (grey line) revealed equation shown above, R = Regression coefficient

The biomass of *P.* sp. MT 1 (and of *P. putida* G7::4/4 and *P. putida* A02) was calculated by means of the equation generated by Manfred Höfle for a different *Pseudomonas* species (*Pseudomonas fluorescens*, strain K), (Höfle, 1976): (mg dw) $l^{-1} = 0.92 + 656.9 \times OD_{578}$.

2.6.2 Cell number

Formalin fixed samples were counted in a Thoma counting chamber under a microscope in phase contrast mode at 40 × magnification. The number of microorganisms (mo) per ml was calculated by multiplication of the average of the counted small quadrates times 2×10^7. The basis for the calculation of the cell concentration is shown here:

mo ml^{-1} = mo per square × 10^3 /chamber area [0.0025 mm^2] × chamber height [0.02 mm]

= mo per square × 2×10^7

For the correlation between optical density and cell number a calibration curve (see Figure 5), was generated.

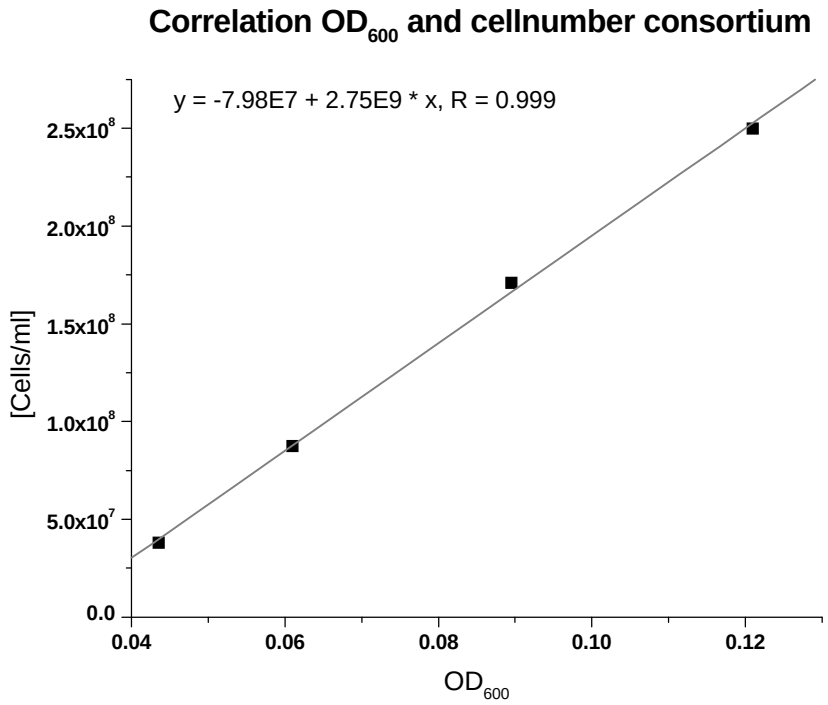

Figure 5: Correlation between OD_{600} and cell number (■) of the 4-CS degrading consortium. Linear fit of data (grey line) revealed equation shown above, R = Regression coefficient.

2.6.3 Optical density

The measurement of the optical density of a cell suspension is and indirect method for the calculation of the cell concentration (Koch, 1961). In this thesis was the optical density measured as the extinction at 600 nm (OD_{600}) in plastic halfmicrocuvettes with 1 cm length in a photospectrometer. The biomass of the bacterial cells (see 2.6.1) and in some experiments the cell number (see 2.6.2) was calculated from OD_{600} measurements. The extinction can be influenced by metabolites produced during the degradation of 4-CS, therefore was the optical density measured with sterile filtered medium as blank. When the sample size was very small water was used as blank. The detection limit of this method lies below an OD_{600} of 0.01; therefore experiments with 4-CS concentrations below 0.1 mM 4-CS were not evaluated.

2.6.4 Data analysis of batch culture results for calculation of growth parameters

To asses the specific growth rate (μ), the doubling time (t_d), the yield and the degradation rate (r_S) of the consortium and of *P*. sp. MT 1, different batch experiments with 4-CS concentrations from 0.1 to 5 mM 4-CS were analysed. The meassured turbidity (2.6.3) (was translated to dry cell mass, to ensure comparability consortium and *P*. sp. MT 1 data was calculated by the same equation ((mg dw) l^{-1} = 0.92 + 656.9 × OD_{578} (2.6.1)) and the decrease of 4-CS (2.8.1) were subject for the analysis. Additionally, it was assessed whether additional HPLC peaks would indicate the occurrence of metabolites like *cis*-dienelactone and protoanemonin.

The cell concentration varies over time according to $dx/dt = \mu x$. This equation can be integrated to x (cell concentration) = x_0 (cell concentration at time zero) $e^{\mu t}$ (e = base of natural logarithm, μ = specific growth rate, t = time) during the exponential growth phase; taking natural logarithms, the equation $\ln x = \ln x_0 + \mu t$, a plot of ln x versus time gives a straight line with slope μ. μ was calculated by graphical data analysis (see Figure 6B).

At least two different growth rates can be calculated from the natural logarithm of the biomass of the batch growth of the consortium on 1 mM 4-CS (see Figure 6). Only the data points corresponding to 4-CS degradation (starting after 28 h, see Figure 6A) were used for estimation of the growth rate. In one hour 0.019 mg new cells generate from 1 mg cells. The doubling rate was calculated with the equation $t_d = \ln 2/\mu$ is 36.5, in 37 h the dry weight of the cells doubled.

The integral yield of the biomass concentration at the end of the exponential phase was calculated. The yield (Y_{XS} = mass of biomass produced per unit mass of substrate consumed) was obtained by applying $Y_{XS} = -\Delta x/\Delta s$ = mass of cells (dw)/mass of substrate. Exemplary the calculation of the yield for the above displayed experiment is shown here: The optical density calculated in biomass minus the biomass at the time point 0 is $t_{96} - t_0$ = 48.235 mg l^{-1} - 5.86 mg l^{-1} = 42.375 mg l^{-1}; divided by 1 mM 4-CS (corresponding to 172 mg l^{-1}) is 0.246. Of 1 g substrate 0.246 g dry weight were produced.

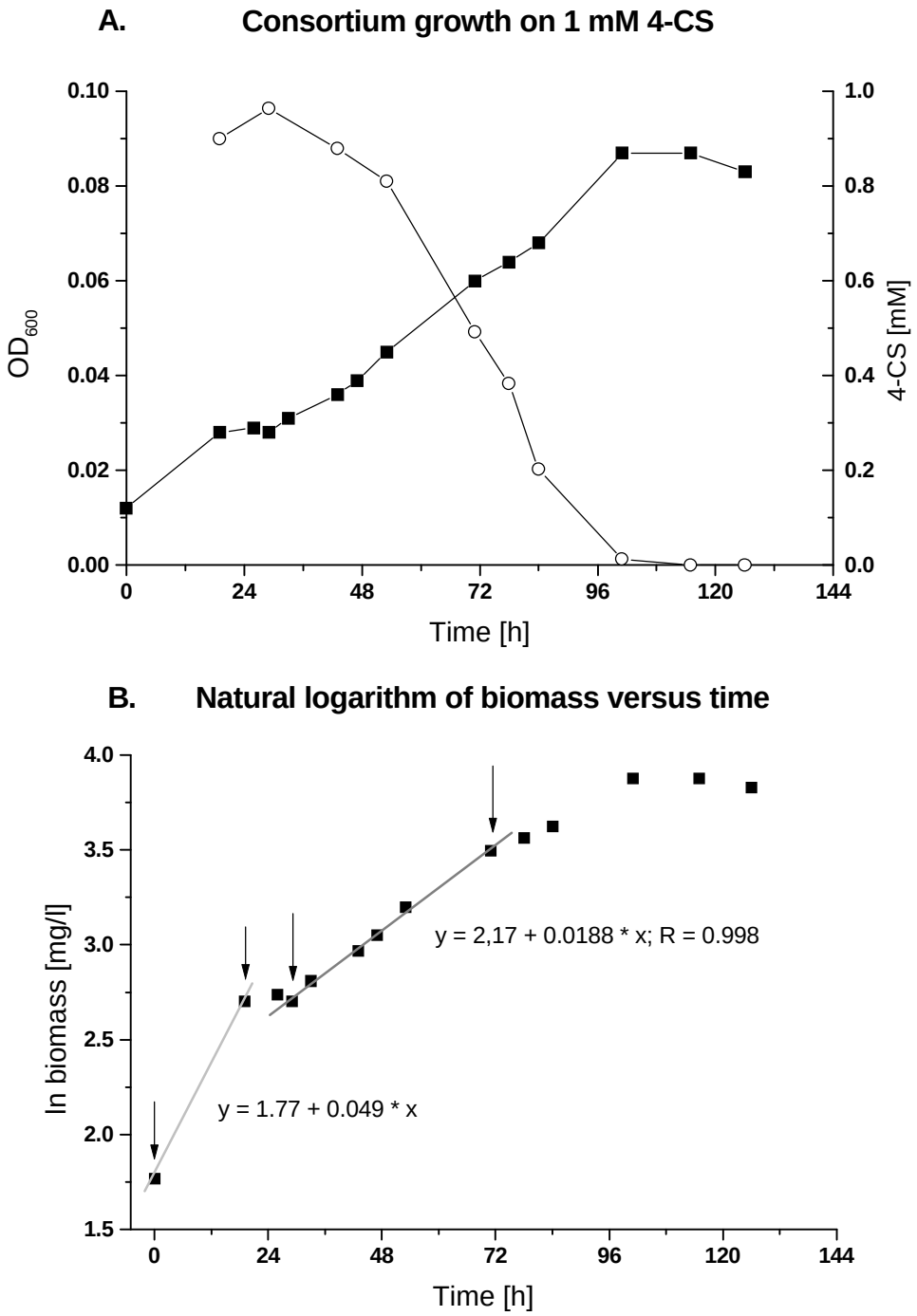

Figure 6: Consortium growth on 1 mM 4-CS and data analysis, A. Optical density (■) and 4-CS concentration (○), B. plot of the natural logarithm of the biomass (■) versus time to estimate the growth rate, linear fit (light grey line) of primary growth, linear fit (grey line) of the exponential growth phase, included data points are indicated by arrows, the equation is shown above

The decrease of the substrate concentration over time can be described as $dS/dt = -r_S x$; (S = substrate concentration, x = cell concentration; r_S = degradation rate, t = time). Solving this equation $r_S = 1/x \times dS/dt$; for small changes $r_S = 1/x \times \Delta S/\Delta t$. The degradation rate was assessed by calculation of the slope between the single data points of the 4-CS decrease and

division of this volumetric rate by the mean cell concentration; $r_S = ((S_{tn2} - S_{tn1})/(t_{n2} - t_{n1})) \times 1/x_{tm2}$. ($S_{tn2}$ = substrate concentration at time point tn2; x_{tm2} = cell concentration at time point tm2). $x_{tm2} = (x_{tn2} + x_{tn1})/2$ The mean rate corresponding to the linear 4-CS decrease was assessed. The degradation rate of the above shown experiment was 0.440 mM 4-CS h^{-1} $(g\ dw)^{-1}$.

2.7 Analysis of enzymatic composition

2.7.1 Preparation of crude cell extracts

Cells were harvested by centrifugation at 12000 *g* (9000 rpm) for 9 min at the end of the logarithmic growth phase, resuspended in 10 ml Tris-HCl (pH 7.5, 50 mM), centrifuged again and resuspended in 1 ml buffer and passed twice through a French pressure cell operating at 10000 lb/in^2. The cell debris was removed by ultra-centrifugation at 100000 *g* (45000 rpm) for 35 min at 4 °C. The clear supernatant is referred to as the cell extract.

The protein concentration was estimated by the method of Bradford with bovine serum albumin as standard (BIO RAD).

Tris-HCl, 50 mM, pH 7.4	Tris-base	6.06	g
	adjust pH with HCl to	7.4	
	in H_2O	1000	ml

2.7.2 Enzymatic assays

The cell extracts were analysed photometrically for potential enzyme activities. Catechol 1,2-dioxygenase was measured by following the formation of muconic acid, the *ortho*-cleavage product of catechol, or 3-chloromuconic acid, the *ortho*-cleavage product of 4-chlorocatechol or 2-chloromuconic acid, the *ortho*-cleavage product of 3-chlorocatechol at 260 nm by a modification of the method of Dorn and Knackmuss (Dorn & Knackmuss, 1978a; Dorn & Knackmuss, 1978b). The following reagents were added to a quartz cuvette: 660 µl 50 mM Tris, (pH 8.0) and 20 mM EDTA; 328 µl distilled water; and 2 µl cell-free extract. The reaction was started by the addition of 10 µl 20 mM catechol or (the corresponding amount less water) 20 µl 10 mM 4-chlorocatechol or 20 µl 10 mM 3-chlorocatechol to a final concentration of 0.2 mM.

Catechol 2,3-dioxygenase activity was measured by following the formation of 2-hydroxymuconic semialdehyde, the *meta*-cleavage product of catechol, or the formation of 5-chloro-2-hydroxymuconic semialdehyde, the *meta*-cleavage product of 4-chlorocatechol at 375 nm. The procedure used was as for chlorocatechol-1,2-dioxygenase activity.

The following extinction coefficients were used to calculate the specific activities: muconic acid at 260 nm = 16,800 l mol^{-1} cm^{-1}, 3-chloromuconic acid at 260 nm = 12,400 l mol^{-1} cm^{-1}, 2-chloromuconic acid at 260 nm = 17,200 l mol^{-1} cm^{-1}, 2-hydroxymuconic

semialdehyde at 275 nm = 36,000 l mol^{-1} cm^{-1} (Sung Bae et al., 1996), 5-chloro-2-hydroxymuconic semialdehyde at 279 nm = 40,000 l mol^{-1} cm^{-1} (Asturias & Timmis, 1993; Sung Bae et al., 1996). The specific enzyme activity is expressed as µmoles of product formed per min and mg of protein.

2.7.3 Differentiation between *meta*- and *ortho*-cleavage enzymes

One disturbance resulted in the accumulation of a substance, which is probably the *meta*-cleavage product of 4-CC. Therefore it was necessary to distinguish between *meta*- and *ortho*-cleavage enzyme activity (see 3.5.1), by differentiation on base of the increased heat-stability of the *meta*-cleavage enzymes. A part of the raw extract was boiled at 50 °C for 10 min, to destroy the *ortho*-cleavage enzymes. Measured activity should now be due to *meta*-cleavage enzymes. A different portion of the raw extract was supplied with 0.01% H_2O_2, which destroys the *meta*-cleavage enzymes, measured activity should now be due to *ortho*-cleavage enzymes.

2.8 Analytical chemistry

2.8.1 Concentration of 4-CS and metabolites (HPLC)

The amount of 4-chlorosalicylate (4-CS) as parameter for the degradative function of the consortium was determined by high pressure liquid chromatography (HPLC) analysis. The substrate and products were quantified by a Waters 712 WISP HPLC equipped with a Macherey-Nagel column (250 × 4 mm) filled with 5 µm Nucleosil particles. The areas (µV s^{-1}) were compared with those from analytical standards. An aqueous solution of 50% (v/v) methanol (MeOH) and 0.1% (v/v) H_3PO_4 in deionised water was used as the mobile phase (flow rate, 1 ml min^{-1}). If the existence of metabolites was indicated by peaks at low retention times, a HPLC run with an aqueous solution of 15% (v/v) methanol and 0.1% (v/v) H_3PO_4 in deionised water was performed to gain a better separation of the metabolites protoanemonin, *cis*-dienelactone and 4-chlorocatechol. The retention times of the metabolites with the different running buffers are summarised in Table 5. Samples of 10 µl were analysed. The column effluent was automatically analysed at 210 and 260/275 nm by a diode array detector (Waters 996).

Table 5: Typical net retention times and characteristic absorption maxima

substance	**running buffer conc. MeOH/H_2O**		**max. wavelength**
	50%/50%	**15%/85%**	
protoanemonin	3.5 min	7.3 min	λmax = 260 nm
cis-dienelactone	3.7 min	8.5 min	λmax = 275 nm
4-chlorocatechol	6.6 min	16 min	λmax = 285 nm
4-chlorobenzoate[a]	17.5 min	min	λmax = 239 nm
2,4-dichlorobenzoate[a]	23.2 min	min	λmax = 238 nm
4-chlorosalicylate	30.6 min	min	λmax = 304 nm

[a]4-chlorobenzoate and 2,4-dichlorobenzoate are minor contaminations of the 4-chlorosalicylate chemical (Pelz, 1999a).

The 4-CS concentration was measured HPLC the measured area translated to concentration by a calibration curve.

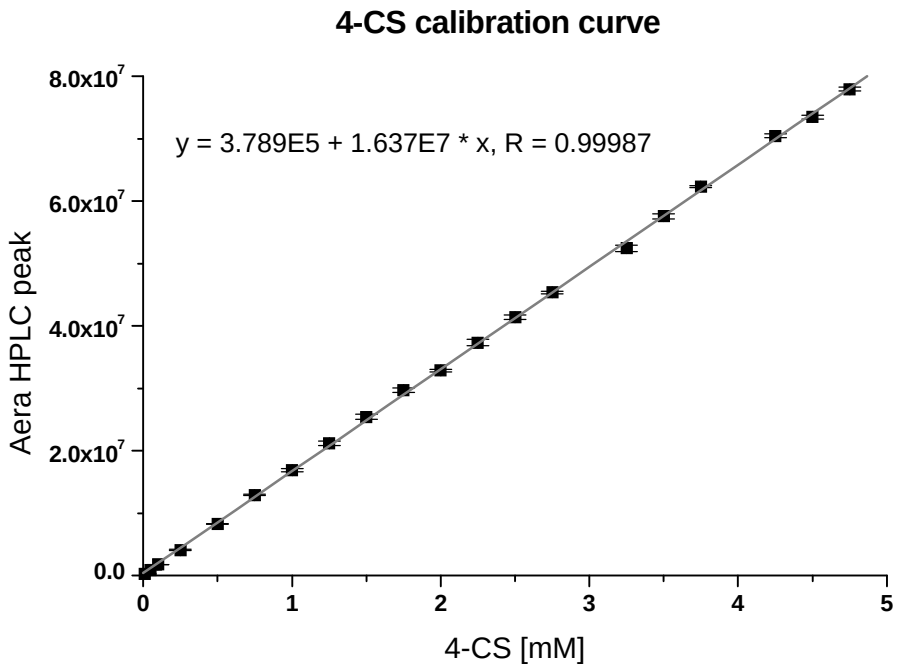

Figure 7: Correlation between 4-CS concentration and HPLC peak area (■). Linear fit of data (grey line) revealed equation shown above, R = Regression coefficient.

Additional HPLC peaks, which indicate the occurrence of metabolites like *cis*-dienelactone and protoanemonin were evaluated by Pelz' calibration curves (Pelz, 1999a).

Table 6: 4-CS concentration and correlating HPLC peak area for lower concentrations; data was fitted with non-linear curve fit, equation not shown

4-CS [mM]	HPLC area	4-CS [mM]	HPLC area
5.00×10^{-5}	1.73×10^{4}	5.00×10^{-3}	1.08×10^{5}
1.00×10^{-4}	2.11×10^{4}	1.00×10^{-2}	2.05×10^{5}
2.50×10^{-4}	2.53×10^{4}	2.50×10^{-2}	5.02×10^{5}
5.00×10^{-4}	3.42×10^{4}	5.00×10^{-2}	9.66×10^{5}
1.00×10^{-3}	4.21×10^{4}	7.50×10^{-2}	1.44×10^{6}

Grey field indicates detection limit.

2.8.2 Dissolved organic carbon (DOC)

One of the influences studied in this thesis was mixed substrate utilisation. One of the substrates was always 4-CS, the other substrate was ethanol, iso-propanol, histidine or $^{1}/_{10}$ NB. The amount of easily utilised carbon sources in the medium reservoir and the reaction vessel was monitored by means of DOC measurement. The observed DOC is a sum parameter, showing the amount of 4-CS and the second carbon source (for example ethanol or histidine). A concentration of 5 mM 4-CS equals a DOC of 360 mg l^{-1}. In the mixed substrate utilisation plots (Chapter 3.3), were the axes always sized due to this correlation. The DOC samples were prepared like the HPLC samples.

An automated analysis system was used. In one channel the total dissolved carbon (DC) is oxidised to CO_2 with a catalysator at 800 °C, in the other channel the inorganic carbon (DIC) is measured with an acid catalysator at 160 °C. The amount of produced CO_2 is detected with an IR-detector. When subtracting the integrated signals of both measurements the amount of the dissolved organic carbon (DOC = DC – DIC) can be calculated. The results are displayed in mg C per ml sample.

2.9 Immunological techniques

The immunological techniques were used for enumeration of the consortium members and of introduced strains. The abundance was determined by direct counts of antibody labelled cells by epifluorescence microscopy.

2.9.1 Antibodies

Cell culture supernatant of a monoclonal antibody and polyclonal sera were used.
Loss of binding ability of antibodies due to differences in the epitopes expressed on cultured organisms was observed previously by Porter et al.. The authors showed that during pure culture studies, a sewage isolate predominantly bound human or goat IgG during exponential phase and little or no binding occurred at stationary phase (Porter et al., 1995). However, in this thesis always good performance of the antibody binding was found observing the 4-CS

degrading consortium members, even when working with samples which were derived from chemostats after one year cultivation. Decreasing fluorescence intensity was always found to be due to bad equipment like old fading dyes or contaminated chemicals.

2.9.1.1 Monoclonal antibodies

The monoclonal antibody #3G8 binds to the surface of *P.* sp. MT 1 cells. Monoclonal antibodies were produced by Faude and Tesar et al. (Faude, 1996; Tesar et al., 1996), the outer membrane proteins from the isolate *P.* sp. MT 1 was used as antigen. The hybridoma cell culture supernatant was named #3G8.

2.9.1.2 Preparation of polyclonal sera

To monitor the fate of competing strains after introduction in the consortium, polyclonal antibodies, reacting against the surface of the strains *P.* sp. B13 SN45P, *P. putida* G7::4/4 and *P. putida* A02, were produced. Rabbits were immunised with formalin fixed pure cultures of the strains.

Sterilised glass tubes containing 5 ml NB medium were inoculated with a colony from a selective plate. The tubes were incubated over night at 30 °C to an OD_{600} of 0.3.

The cells were centrifuged at 6000 *g* (7000 rpm) for 10 min, the supernatant discarded and the cells resuspended in buffer (PBS). This procedure will be referred to as wash step. The wash step was repeated 2 times. 270 µl of 36.5% formalin were added to 5 ml suspension, then the cells were washed 3 times. The OD_{600} of the suspension was adjusted with PBS to 0.3, five aliquots of 1 ml were stored at -70 °C.

One aliquot was mixed with an equal volume of incomplete Freud's adjuvant (Sigma) and the emulsion injected subcutanously into a female rabbit. Four booster injections were given at intervals of three weeks and the animal was bled after 15 weeks. The sera were stored at -20 °C. The gained serum was used directly and tested for cross-reactivity against the other community members and isolates.

2.9.1.3 Polyclonal sera

The double labelling indirect immunofluorescence technique which was applied afforded polyclonal sera. Polyconal sera reacting against the consortium members were produced by Tesar and colleagues (Faude, 1996; Frech, 1996; Tesar et al., 1996), and the polyclonal sera against the competitors were produced as described above (see 2.9.1.2 and Table 7).

Table 7: Polyclonal antibodies used in this thesis

strain	taxonomy	polyclonal antibody
MT 1	*Pseudomonas* sp.	#200
MT 2	*Empedobacter brevis*	#201
MT 3	*Achromobacter xylosoxidans*	#202
MT 4	*Pseudomonas* sp.	#170
B13 SN45P	*Pseudomonas* sp.	#AR 1
G7::4/4	*Pseudomonas putida*	#AR 2
A02	*Pseudomonas putida*	#AR 3

2.9.2 Indirect immunofluorescence membrane filter enumeration

Indirect immunofluorescence (IIF) is a valuable tool to enumerate community populations (Faude & Höfle, 1997). IIF is indirect staining with unconjugated antibodies against specific cellular determinants, detecting these primary staining antibodies with second, fluorochrome-conjugated, frequently polyclonal anti-antibodies. A target-specific polyclonal or monoclonal primary antibody is first bound to its target and then detected by a fluorescently labelled polyclonal secondary antibody raised against the immunoglobulin of the primary antibody. The cellular determinants are on the surface of the bacterial cells, therefore staining results in a ring fluorescence.

To avoid miscalculations of the consortium populations, which were less abundant, a double-staining technique was used in this thesis. Four or 5 parts of each sample were stained. In each sample all cells were labelled with a blue DNA stain, the generally most abundant strain *P.* sp. MT 1 was labelled with a green fluorescent fluorochrome and one of the other consortium members or the competitor were labelled with a red fluorochrome. In one part of each sample all antibodies were applied, to observe, if unstained cells can be detected, which would indicate contaminating cells in the sample/chemostat.

Indirect immunofluorescence was carried out by staining the sample in liquid solution, followed by fixation of the cells on a polycarbonate filter using a vacuum filtration set, embedding of the filter on glass slides and final counting of the labelled cells by epifluorescence microscopy.

2.9.2.1 Staining and embedding protocol

All solutions (for example deionised water, PBS, PBS/T, antibody solutions or conjugate solutions), which were used for immunofluorescence methods, were sterile filtered (0.2 µm pore size, Sartorius) before use.

Bacterial cells were fixed with formaldehyde after sampling, stained with a DNA stain and fluorescent antibodies as previously described (Faude & Höfle, 1997), in 1.5 ml Eppendorf tubes and finally immobilised on black polycarbonate filters (0.2 µm pore size, 25 mm diameter, Costar Corp.).

The staining protocol was performed as described at room temperature:
For each sample four or five different staining combinations were prepared:

- DTAF (dichlorotriazinylamino-fluorescein) and #3G8 (anti-MT 1) and Cy 3 (carboxymethylindocyanine 3) and #201 (anti-MT 2)
- DTAF and #3G8 (anti-MT 1) and Cy 3 and #202 (anti-MT 3)
- DTAF and #3G8 (anti-MT 1) and Cy 3 and #170 (anti-MT 4)
- DTAF and #3G8 (anti-MT 1) and Cy 3 and #AR1 or #AR2 or #AR3 (anti-competitor) - in the specific experiments
- DTAF and #3G8 (anti-MT 1) and Cy 3 and #201, #202, and #170 and #AR1 or #AR2 or #AR3 (anti-competitor) in the specific experiments

10 µl sample were suspended in 600 µl deionised water with 1% (v/v) Roti block (Roth) (H_2O/R) in a 1.5 ml blue, sterilised tube (Eppendorf). 100 µl of the monoclonal antibody #3G8 (1:2 diluted in H_2O/R) and 100 µl polyclonal antiserum #201 (1:100 diluted in H_2O/R) or 100 µl polycolonal antiserum #201 and/or #202, #170, #AR1, #AR2 or #AR3 (1:200 diluted in H_2O/R) were added to incubate the cells on a thermomixer for 60 min.
This results in a final dilution of the monoclonal antibody (1:16), of the polyclonal antibody #201 (1:800) and of the other polyclonal antibodies (1:1600).
100 µl of the DTAF-conjugate solution (1:80 diluted in H_2O/R) and 200 µl of the Cy 3 conjugate solution (1:130 diluted in H_2O/R) were added and incubated on a thermomixer for 60 min.
This resulted in a final dilution of the DTAF (1:900) and of the Cy3 (1:740) conjugated antibody.

Conjugate solution**:**	DTAF (anti-mouse):	conjugated goat anti-mouse (lgG + lgM (H+L) (number 115-015-068), Dianova)
	Cy 3 (anti-rabbit):	sheep anti-rabbit (IgG, whole molecule, (C-2306), Sigma)

50 µl DAPI (4'.6-diamidino-2-phenylindole, dilactate, Polysciences) (1 µg ml^{-1}) solution was added and incubated for 5 min.

The filtration set was prepared by application of a nitro-cellulose filter (0.45 µm, 25 mm diameter, Sartorius) wetted with sterile, filtered water on the glass frit. A wet nucleopore polycarbonate filter was laid on top. The filter set-up was finished by application of the glass tubing by means of a clamp.
3 ml H_2O/R were added for prewetting of the filter set. The sample of the Eppendorf tube (about 1.3 ml) was added, mixed by sucking in and out liquid with the pipet tip, and the set was drained. Three times the cells were rinsed by application of 3 ml H_2O/R, incubation for 5 min and draining.

The polycarbonate filters were transferred to glass slides, covered with the mounting medium Moviol (Hoechst Corp.) containing 1% DAPCO (1,4-diazabicyclo[2.2.2.] octane; Sigma Corp.) to prevent fading, then a cover slip was applied and sealed with nail polish. The slides were stored at 4 °C.

Moviol embedding solution:	Moviol	2.4	g
	Glycerol	6	g
	H_2O	6	ml
	Tris HCl (0.2 M, pH 8.5)	12	ml
	DAPCO	1	%
PBS/T (PBS and 0.05% Tween 20):	PBS	1000	ml
	Tween 20	0.5	g
Tris-HCl (0.2 M, pH 8.5):	Tris	24.22	g
	adjust pH to	8.5	
	in H_2O	1000	ml

2.9.2.2 Epifluorescence counting

The microscopic observations and photomicrographs were done with an epifluorescence microscope (Auxiophot, Carl Zeiss, Oberkochen, Germany), with the objektive Neofluar 100×/1.3 oil and the ocular Pi 10×/25 with counting net and filtersets for DAPI, Cy 3 and DTAF.

Table 8: Dyes used for immunofluorescence and detection systems

dye	excitation	emission	excitation filter	chromatic beam splitter	emission filter
DTAF	480 nm	520 nm	BP 480/30	505	BP 535/40
Cy 3	550 nm	575 nm	BP 545/30	565	BP 610/75
DAPI	366 nm	> 420 nm	BP 360/40	400	LP 420

Between 200-500 cells were counted on different spots on the filter and the results were expressed as percents of DTAF and of Cy 3 stained cells of the total cell number. The average abundance of one consortium member was calculated each time from at least two times counting 100 to 200 cells, but the standard deviation was not calculated every time. The mean value of the two countings was calculated. It was tested, if the sum of the relative abundance of all consortium members (and the competitor) added up to 100% in abundance, a lower value would indicate contaminating cells.

The absolute abundance of the labelled bacteria was determined by multiplying the relative abundance with the total cell counts determined by counting in the Thoma chamber. Digital pictures were made with the same microscope supplied with a ccd camera (Intas) and the software Image Pro II.

3 Results

Figure 8: Optical visible influence of disturbances upon the consortium maintained in continuous culture

The results chapter is structured in two parts. Firstly basic consortium parameters, which were assessed in batch culture studies, are introduced and compared to the basic parameters of the primary degrader *P.* sp. MT 1 (Chapter 3.1). Then the consortium under undisturbed and disturbed conditions maintained in continuous culture, is presented (Chapter 3.2-3.5). Figure 8 shows the colouration of the continuous culture as a result of the influence of different disturbances upon the consortium.

3.1 Growth with different 4-CS concentrations in batch culture of the 4-membered consortium and *Pseudomonas* sp. MT 1

Characteristic growth parameters of the consortium growing in batch cultures were assessed to define control parameters and compared to those of *P.* sp. MT 1. It was expected that the growth parameters of the consortium would differ from those of the main degrader *P.* sp. MT 1, as differences in kinetic parameters between a consortium and its main degrader were previously observed (De Souza et al., 1998; Feigel & Knackmuss, 1993).

Cultures were grown in the presence of 4-chlorosalicylate from 0.1 mM to 5 mM (2.5.3.2). Inoculum of the consortium was derived from a continuously growing culture, whereas *P.* sp. MT 1 was inoculated from a batch culture harvested during late exponential growth. In all experiments turbidity (2.6.3) was meassured (and translated to biomass (2.6.1)) and the decrease of 4-CS was meassured (2.8.1). Additionally, it was assessed whether metabolites like *cis*-dienelactone and protoanemonin accumulated during growth.

Typical growth curves of the consortium and *P.* sp. MT 1 on 1 mM 4-CS and on different 4-CS concentrations are shown in Figure 9 and Figure 10, respectively. These and additional experiments were used to calculate the characteristic kinetic parameters. The yield was calculated by division of biomass concentration at the end of the exponential growth phase by 4-CS concentration; the specific degradation rate was calculated by division of the slope of 4-CS decrease by biomass; and the growth rate was calculated by estimation of the slope of the natural logarithm of the biomass at the time corresponding to 4-CS decrease. One exemplary set of data analysis is described in Chapter 2.6.4.

3.1.1 Batch growth of consortium and *P.* sp. MT 1 on 1 mM 4-CS

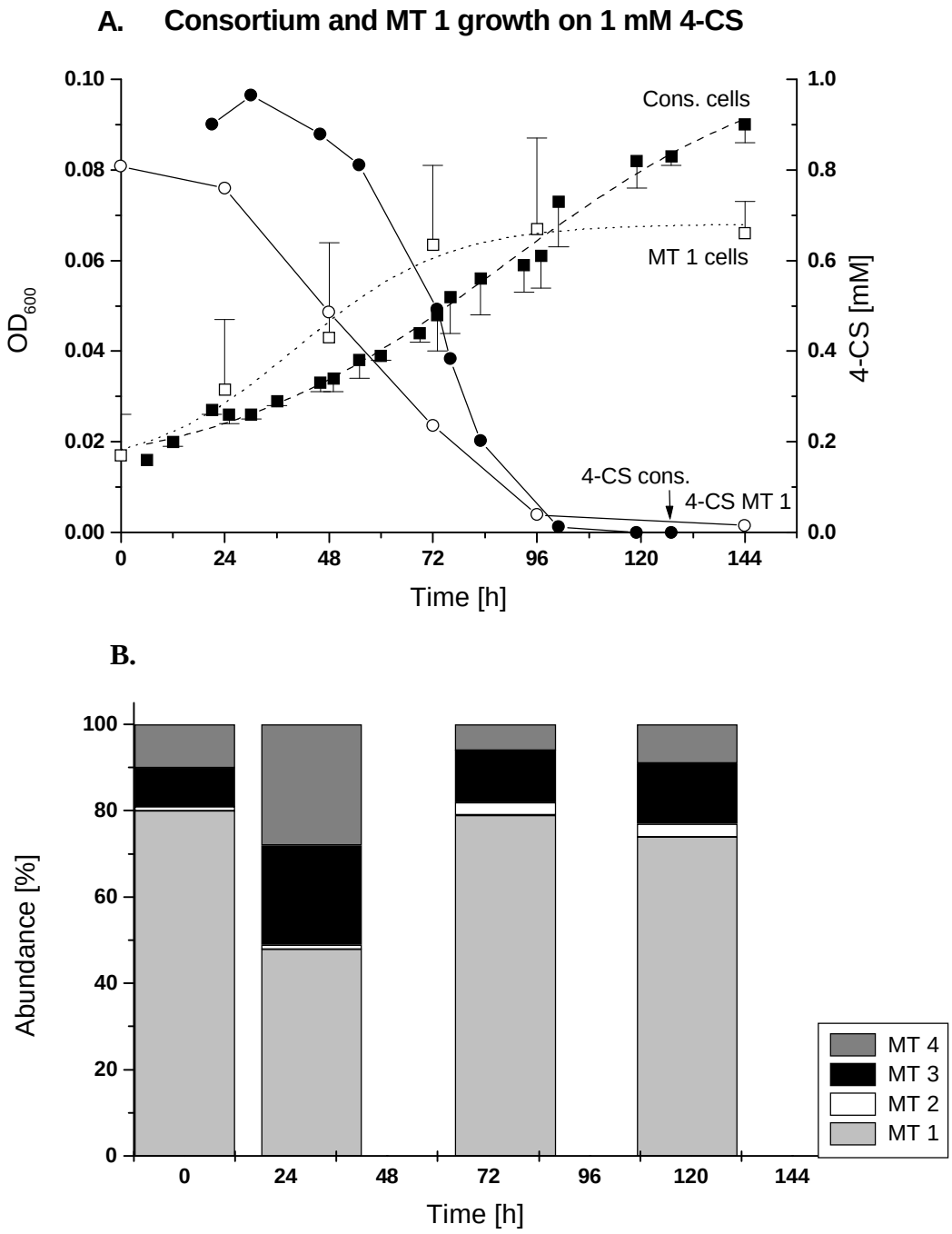

Figure 9: A. 4-chlorosalicylate (4-CS (●)) degradation and cell concentration (■) of the consortium and 4-CS (○) degradation and cell concentration (□) of *P.* sp. MT 1 and B. absolute abundance consortium members, when grown in a batch culture with 1 mM 4-CS as sole source of carbon and energy. The substrate concentration and absolute abundance were acquired in one experiment. The bars indicate standard deviation of the cell concentration (s.d. consortium n = 3, only negative bars displayed), (s.d. *P.* sp. MT 1 n = 2, only positive bars displayed). The dashed and the dotted line displays the sigmoidal curve fit for the consortium and for *P.* sp. MT 1, respectively.

The growth of the consortium and of *P.* sp. MT 1 in batch culture on 1 mM 4-CS is shown in Figure 9A. The growth rate of the consortium with 1 mM 4-CS was 0.019 h^{-1} (at the time of 4-CS degradation, that is between 24 h and 96 h). The growth rate corresponds to a doubling time of 37 h. The 4-CS degradation rate of the consortium was 0.440 mM h^{-1} $(g\ dw)^{-1}$. In the first 24 h of growth only minor 4-CS concentration decrease, but increase in cell concentration at a growth rate of 0.05 h^{-1} was observed. The consortium composition, as shown in Figure 9B, changed during batch growth; *A. xylosoxidans* MT 3 and *P.* sp. MT 4 became more abundant at the beginning of the exponential phase. The increase in cell concentration during the first 24 h is based on the growth of these two strains (see Figure 9A + B). At the end of the exponential growth phase declined the abundance of *P.* sp. MT 4 and the consortium reached this consortium composition: MT 1 = 74%, MT 2 = 3%, MT 3 = 14% and MT 4 = 9%, respectively. The growth rate of *P.* sp. MT 1 was 0.018 h^{-1}, this corresponds to a doubling time of 39 h. The 4-CS degradation rate of *P.* sp. MT 1 was 0,234 mM h^{-1} $(g\ dw)^{-1}$. These results show that the consortium displayed a similar growth rate, however reached a higher degradation rate than *P.* sp. MT 1.

3.1.2 Batch growth of consortium and *P.* sp. MT 1 on different 4-CS concentrations

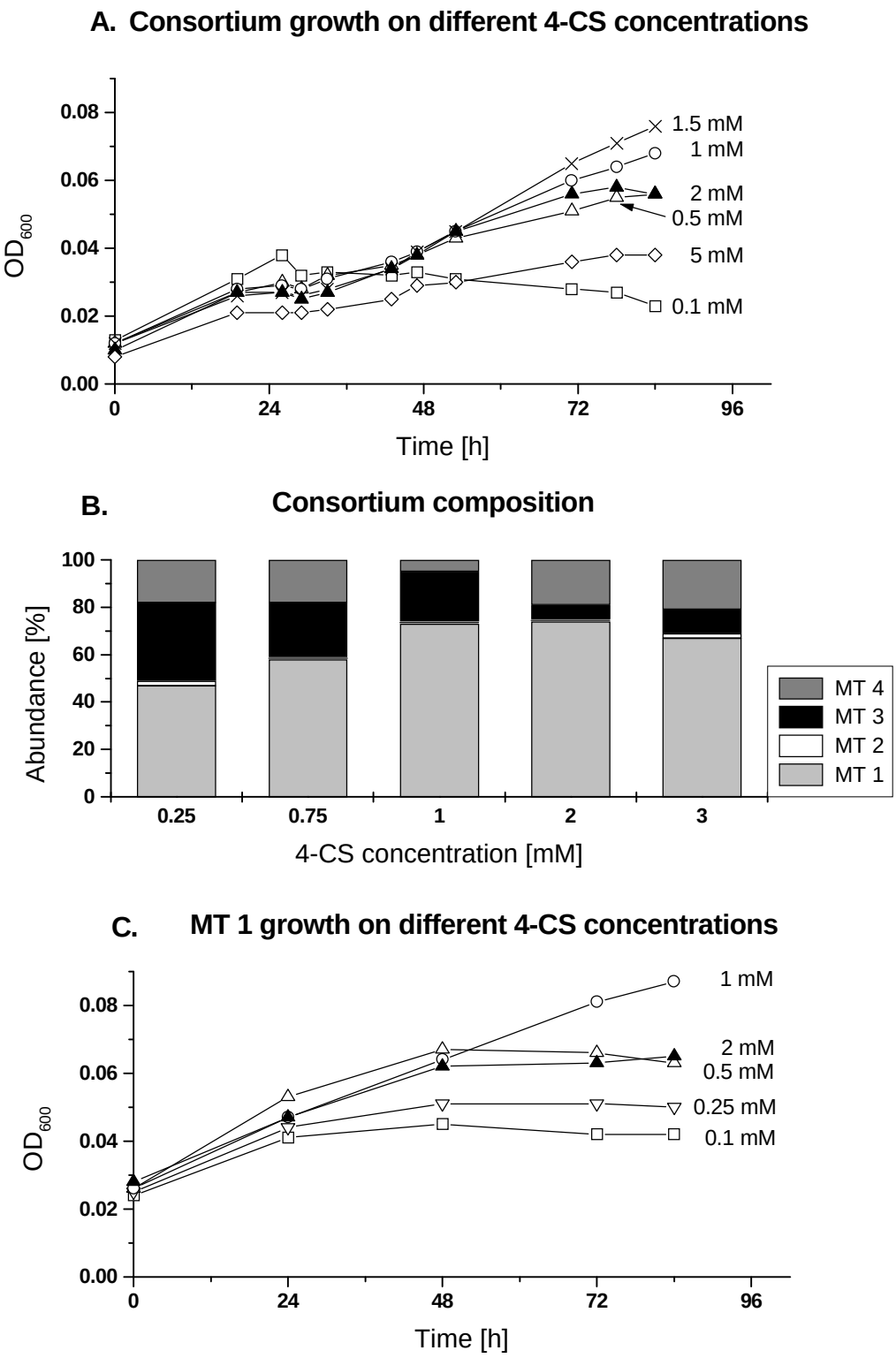

Figure 10: Optical density of the batch growth of A. the consortium and C. *P.* sp. MT 1 on minimal medium with 0.1 (□), 0.25 (▽), 0.5 (△), 1 (○), 1.5 (×), 2 (▲) and 5 (◇) mM 4-CS; B. consortium composition at the end of the exponential growth phase at different 4-CS concentrations.

The cell concentration and structure of the consortium and *P.* sp. MT 1 cells incubated in batch culture with varying substrate concentrations is shown in Figure 10. Increasing 4-CS concentrations (up to 1 mM for *P.* sp. MT 1 and up to 1.5 mM for the consortium) resulted in increasing optical density. Higher substrate concentrations (2 mM and 5 mM 4-CS) resulted in lower optical densities (compared to the 1 mM and 1.5 mM experiments). After 84 hours for the consortium and after 72 hours for *P.* sp. MT 1 a brown intermediate accumulated in the reaction vessels with 2 mM and 5 mM 4-CS concentration, presumably the auto-oxidation product of 4-chlorocatechol. The occurrence of this metabolite is further discussed in Chapter 4.2.2.

Consortium composition was determined at the end of the exponential phase and differed after growth on different substrate concentrations. The abundance of *P.* sp. MT 1 increased from the concentration 0.25 to 1 mM from 45% to 70%, at 2 and 3 mM the abundance of *P.* sp. MT 1 remained at 70%. At 4-CS concentrations of 0.25, the abundance of the consortium member *A. xylosoxidans* MT 3 was 30%.

3.1.3 Specific growth rate of consortium and *P.* sp. MT 1

The specific growth rates acquired by the single experiments are plotted versus the substrate concentration in Figure 11.

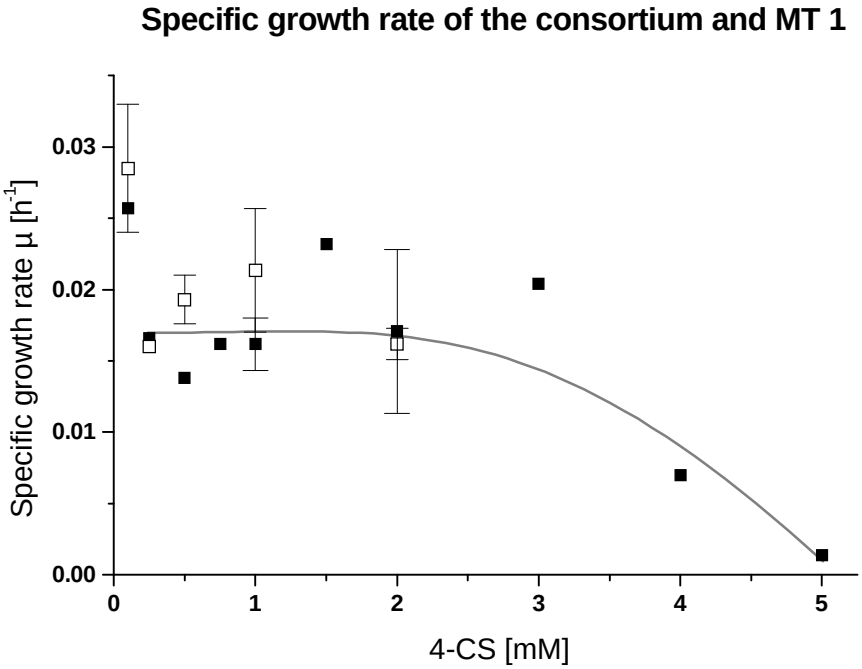

Figure 11: Specific growth rate of the consortium (■) and *P.* sp. MT 1 (□) calculated from the results of various batch experiments with different 4-CS concentrations. Hand-drawn approximation of non-linear curve fit (grey line) of the data points belonging to the consortium experiments

The data of the consortium and of *P*. sp. MT 1 displayed a high scatter. During balanced growth, the growth rate was related to the concentration of growth-limiting substrate. The most frequently-used expression relating growth rate to substrate concentration is the Monod equation. When growth is inhibited by high substrate concentrations, extra terms can be added to the Monod equation to account for these effects. Several other kinetic expressions have been developed for cell growth (Bailey & Ollis, 1986; Moser, 1985). These equations can not be applied for the above shown data because in the limited part of the curve no increase at small substrate concentrations was observed. The general trend, which can be extracted from Figure 11, is that the specific growth rate of the consortium between 0.5 and 3 mM 4-CS remained stable around the mean 0.018 h^{-1} and then decreased with higher substrate concentration. The growth rate of *P*. sp. MT 1 between 0.5 and 2 mM 4-CS remained stable around the mean 0.019 h^{-1}.

3.1.4 4-CS degradation rates of consortium and *P.* sp. MT 1

The average degradation rates of the consortium and *P*. sp. MT 1 were plotted versus the substrate concentrations in Figure 12.

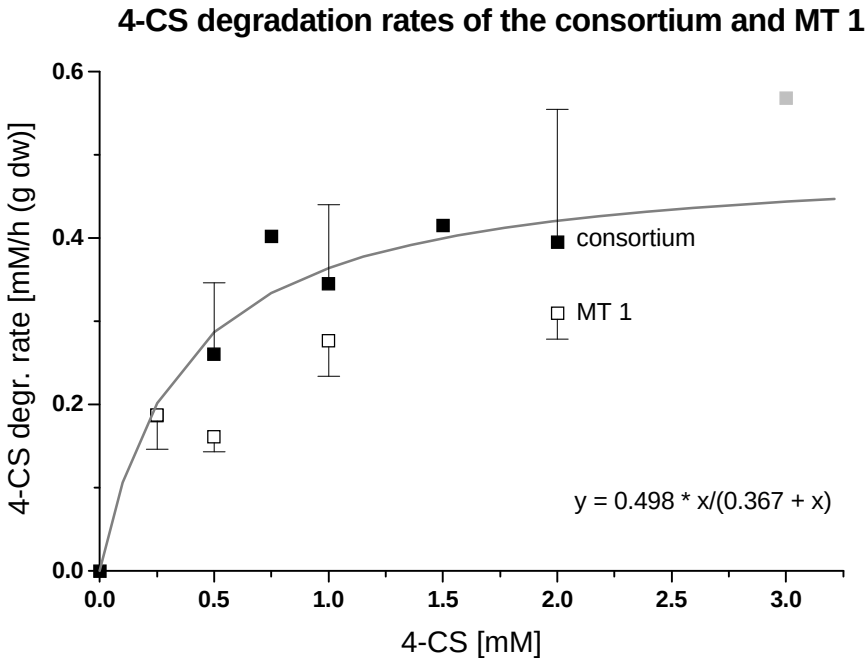

Figure 12: 4-CS degradation rates of the consortium (■, outlier ■), and *P*. sp. MT 1 (□) plotted versus the starting concentrations of 4-CS, Bars indicate standard error of the mean (SEM) (n = 2 - 3), positive bars SEM for the consortium, negative bars SEM for *P*. sp. MT 1; Non-linear curve fit with Monod equation (grey line) revealed equation shown above.

The consortium had higher degradation rates than *P*. sp. MT 1. The average degradation rate for *P*. sp. MT 1 was 0.234 mM $h^{-1}(g\ dw)^{-1}$. The average degradation rate for the consortium between 0.5 and 2 mM 4-CS was 0.364 mM $h^{-1}(g\ dw)^{-1}$. Omitting the outlier at 3 mM 4-CS

concentration, a non-linear fitting with a modification of the Monod equation $r_S = r_{Smax} \times [S]/K_S + [S]$ revealed the equation shown in Figure 12. According to this equation is $r_{Smax} = 0.498$ mM $h^{-1}(g\ dw)^{-1}$ and $K_S = 0.367$ mM (K_S is the substrate concentration at which the growth rate is half maximal). The degradation rate of the consortium and of *P*. sp. MT 1 increased at substrate concentrations below 1 mM, then remained stable. The average degradation rate of the consortium was 35% higher than the degradation rate of *P*. sp. MT 1.

3.1.5 Growth yields at different substrate concentrations

The average substrate yields of the consortium and *P*. sp. MT 1 are plotted versus the substrate concentrations in Figure 13.

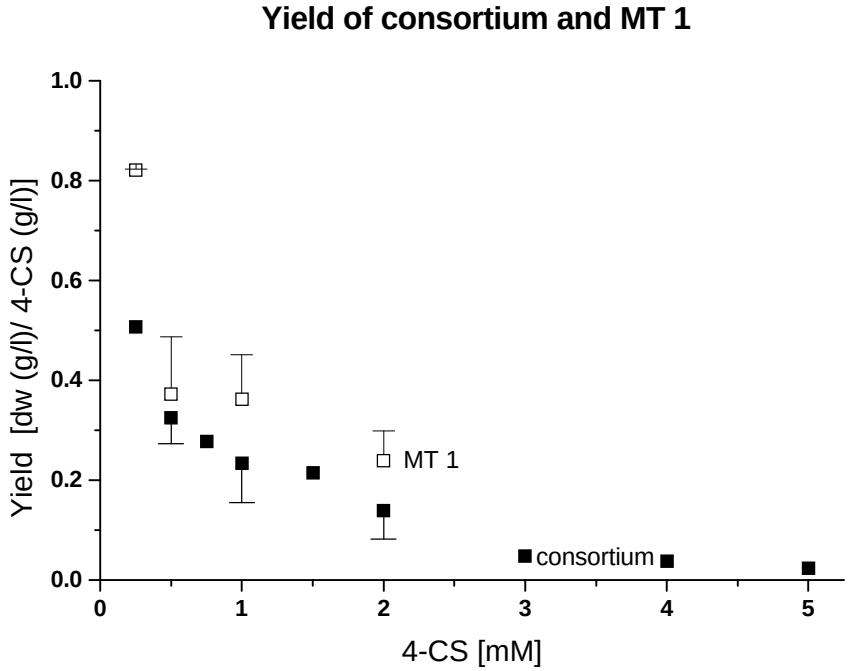

Figure 13: Batch culture yields of the consortium (■) and *P*. sp. MT 1 (□) plotted versus the starting concentrations of 4-CS. Bars indicate standard error of the mean (SEM) (*n* = 2 - 5), positive bars SEM for *P*. sp. MT 1, negative bars SEM for the consortium.

The average consortium yield between 0.5 and 1.5 mM 4-CS was 0.263, and the average yield of *P*. sp. MT 1 between 0.5 and 1 mM was 0.367. The general trend, which can be extracted from Figure 13, is that the yields of *P*. sp. MT 1 and the consortium are stable between 0.5 to 1 mM for *P*. sp. MT 1 and between o.5 to 1.5 mM for the consortium. Further increasing substrate concentrations result in the accumulation of a brown metabolite and decreasing yields of the consortium and of *P*. sp. MT 1.

3.1.6 Comparison of kinetic parameters of consortium and its primary degrader *P.* sp. MT 1

Table 9: Comparison of kinetic parameters of consortium and its primary degrader *P.* sp. MT 1

parameter	consortium	MT 1
average growth rate	0.018 h^{-1} (39 h)	0.019 h^{-1} (36 h)
average degradation rate	0.364 mM $h^{-1}(g\ dw)^{-1}$	0.234 mM $h^{-1}(g\ dw)^{-1}$
K_S	0.367 mM	nd
average yield	0.263	0.367

The comparison of kinetic parameters, summarised in Table 9, shows that the values for *P.* sp. MT 1 and the consortium were similar. The parameters for the growth rate and the yield of *P.* sp. MT 1 were higher than the consortium. *P.* sp. MT 1 showed a higher average yield than the consortium. Only the degradation rate of the consortium was 20% higher compared to the value of the primary degrader. This is also apparent in Figure 9A, where the consortium 4-CS degradation graph is steeper than that of *P.* sp. MT 1.

In summary, small differences between the consortium and *P.* sp. MT 1 were observed, but these differences are not significant enough to confirm a better kinetic composition of the consortium compared to its primary degrader.

3.2 Consortium growth in continuous culture and average consortium composition under undisturbed conditions and the role of consortium member *Pseudomonas* sp. MT 4

In the previous chapter kinetic parameters for the consortium growth in batch culture were assessed, additionally the consortium behaviour in continuous culture under undisturbed conditions was defined. This was necessary because all chemostats must have the same undisturbed steady state conditions prior to application of the disturbances to be able to observe the influence of the disturbances.

Growth of the consortium in continuous culture with 5 mM 4-CS (undisturbed continuous culture) is shown in Figure 14. No significant changes were observed in the consortium structure, as shown by the relative abundance of the consortium members (Figure 15). This control showed the fluctuations based on the handling of the reactors, so these changes were not due to applied disturbances. The dilution rate at the start of the continuous culture was $D = 0.02\ d^{-1}$. This rate was stepwise increased until it finally reached $D = 0.1\ d^{-1}$ as described in 2.5.5.3. The increasing dilution rate resulted in an increase of the cell number between day 0 to day 7, because the higher amount of carbon source results in a higher cell concentration, as the chemostat is carbon limited. In this experiment the optical density and cell number did not vary much after 7 days. 4-CS accumulation was usually not observed during these experiments. Neither did metabolites like 4-chlorocatechol, *cis*-dienelactone or protoanemonin accumulate. The dissolved organic carbon (DOC) concentration was at $30\ mg\ C\ l^{-1}$, this is the background value. The average consortium structure throughout the experiment was MT 1 = 75 ± 5%, MT 2 = 1 ± 0.3%, MT 3 = 19 ± 3% and MT 4 = 5 ± 3%.

3.2.1 Continuous culture of consortium with 5 mM 4-CS, undisturbed control

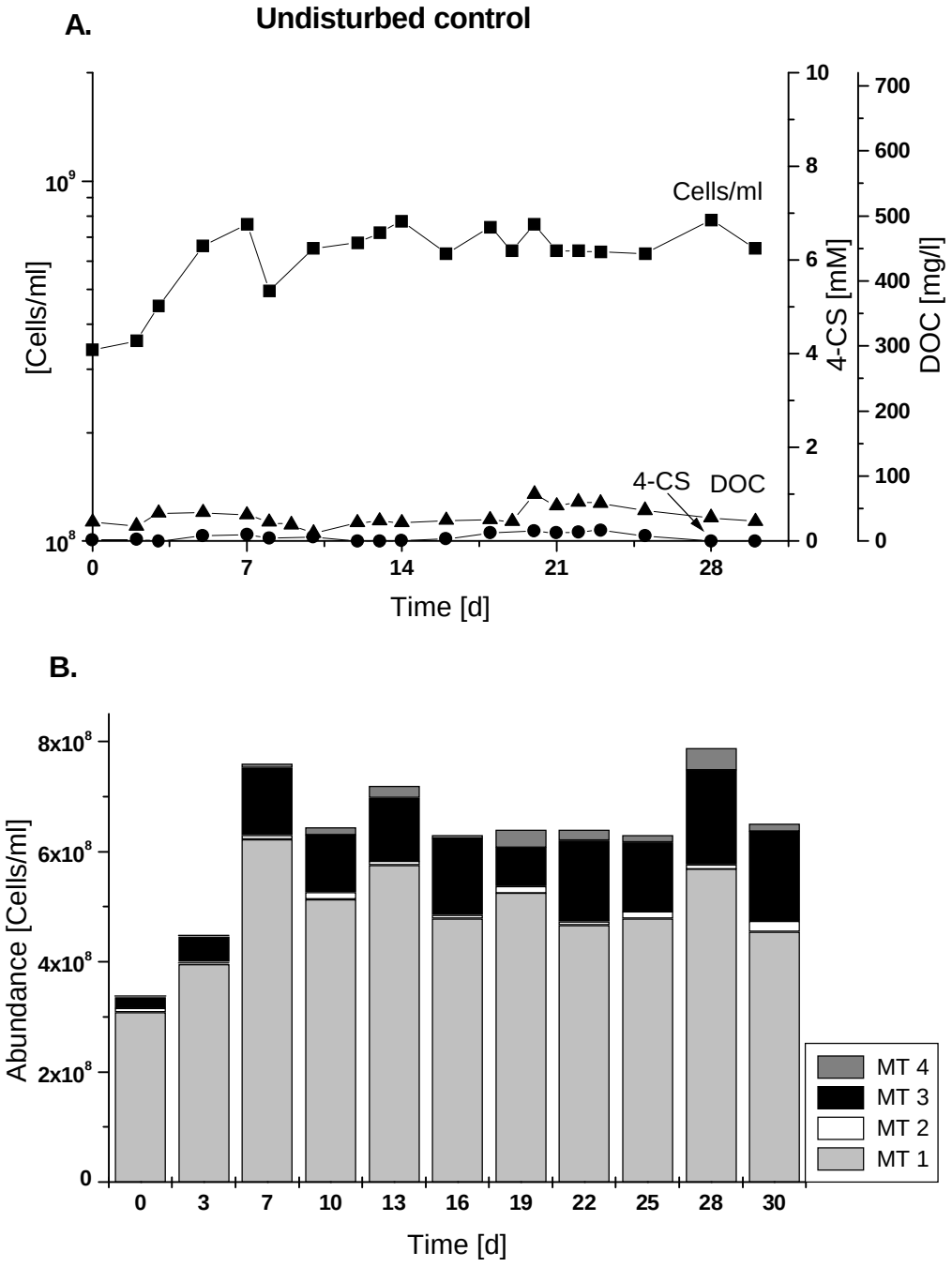

Figure 14: Undisturbed consortium A. Cell number (■), 4-CS concentration (●) and DOC (▲), B. absolute abundance of consortium members

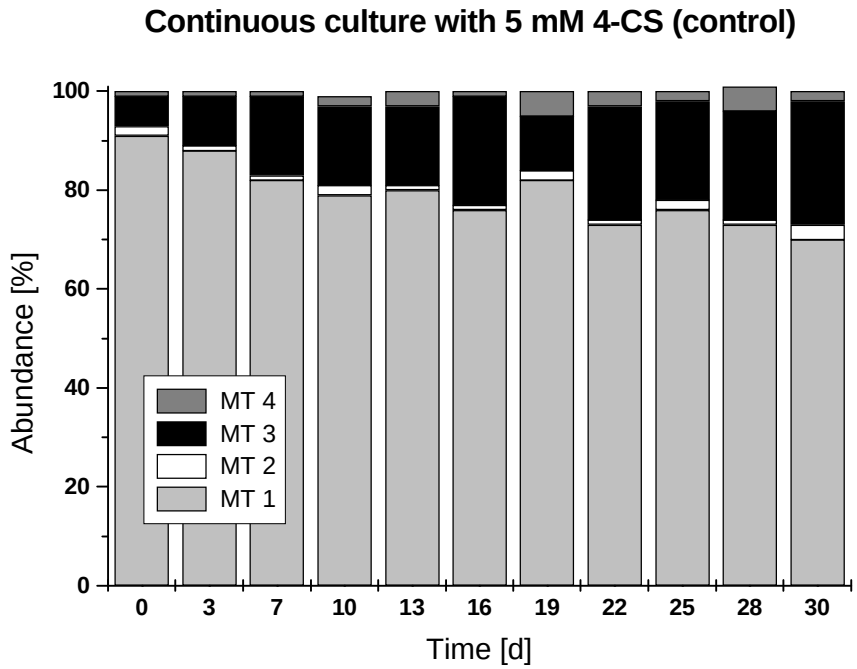

Figure 15: Undisturbed consortium; relative abundance of consortium members

3.2.2 Statistical analysis of all experiments to access the average consortium composition

The average consortium composition of the undisturbed consortium was assessed by statistical analysis of all undisturbed experiments. These were steady state conditions of undisturbed continuous culture experiments or steady state conditions prior to application of a disturbance. Additionally was the consortium composition under steady-state conditions of the undisturbed controls of fed-batch culture growth applied. In another statistical analysis was made use of the consortium composition at the end of the exponential phase of batch culture growth. The cells were counted and the consortium composition was calculated as described previously (see 2.9.2). One example of the mean value and the standard deviation of one sample is shown in Figure 16.

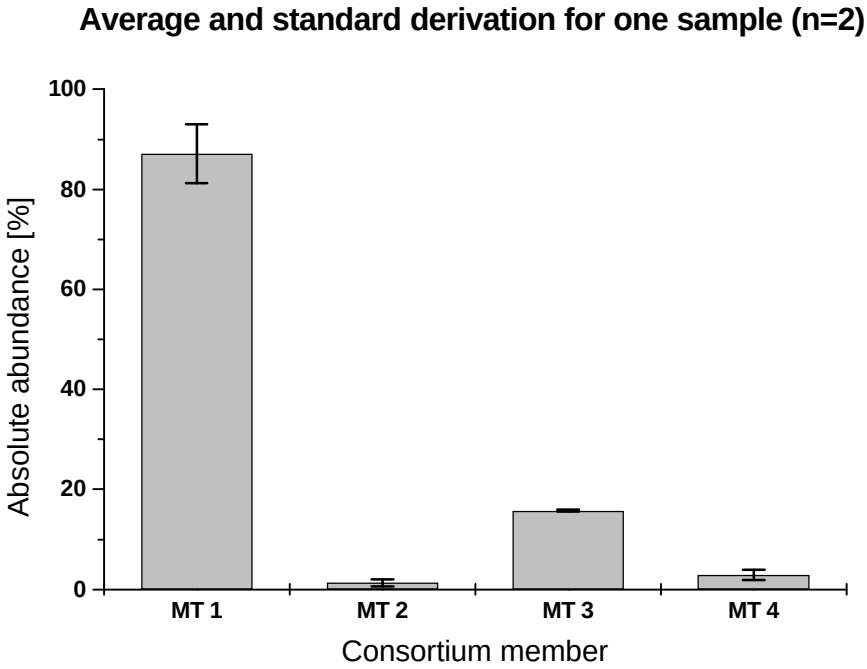

Figure 16: Absolute abundance and standard deviation for the counting of the consortium members of one sample (control, day 16, Chapter 3.3.2)

The result of the calculation of the undisturbed consortium composition could be divided into two expected consortium compositions. One for the batch cultures:
MT 1 = 74 ± 1.2%, MT 2 = 2 ± 0.7%, MT 3 = 16 ± 2.3% and MT 4 = 7 ± 1.0%, (n = 9), and one for the steady-state conditions of fed-batch culture and the continuous culture:
MT 1 = 76 ± 1.3%, MT 2 = 2 ± 0.7%, MT 3 = 19 ± 1.2% and MT 4 = 3 ± 1.2%, (n=35);

3.2.3 Role of consortium member *P.* sp. MT 4

Here one experiment showing the growth of the consortium in fed-batch culture without carbon source is presented. This experiment resulted in an enhanced abundance of *P*. sp. MT 4. The result of this experiment is necessary in order to interpret the role of the consortium member *P*. sp. MT 4 in the following continuous culture experiments.
The reaction of the consortium to the omission of a carbon source in the medium was studied. The consortium was subjected to a fed-batch culture as described in 2.5.4. The undisturbed fed-batch control is shown in Figure 17. The experiment conditions differed from the optimal composition in two points. Firstly was the medium feeding rate inconsistent. The medium was fed at a rate of 2 ml h^{-1} for the first 4 h, then the flow was stopped for 20 h (batch growth phase) and started again at a flow rate of 1.5 ml h^{-1}. Secondly was the consortium composition not optimal. Only approximately 60% total abundance were reached, due to the existence of an invading strain. Additionally, the consortium composition consisted primarily of

P. sp. MT 1, however under the influence of 5 mM 4-CS feeding (the fed-batch control) the consortium composition started to recover (see Figure 17B).

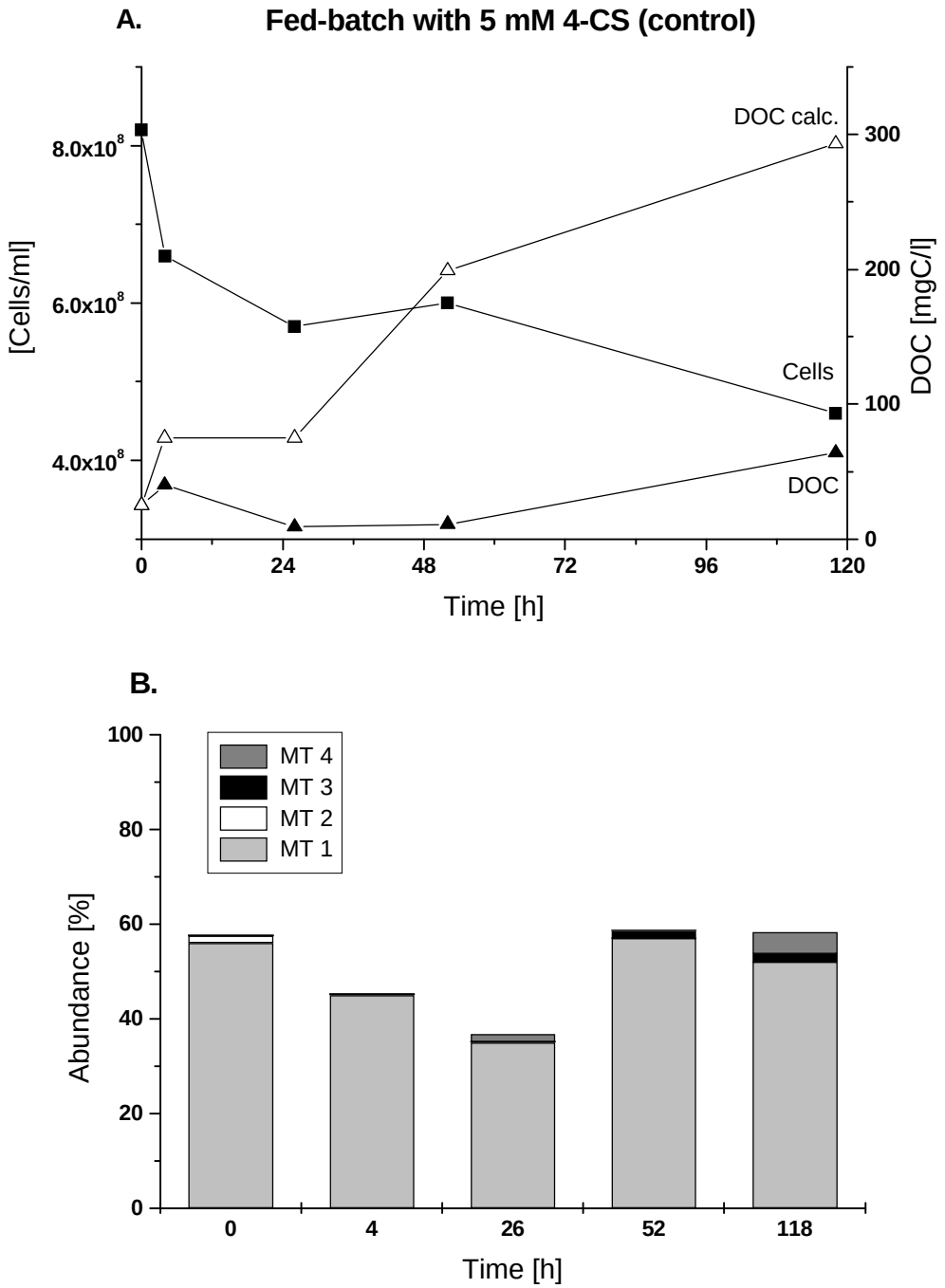

Figure 17: Fed-batch culture of the consortium with 5 mM 4-CS, A. Cell number (■) and measured DOC (▲), calculated DOC (△) without degradation, B. absolute abundance of the consortium members.

Nevertheless, the reaction of the consortium to the omission of a carbon source in the feedstock is shown in Figure 18. In the first 24 h the DOC increased, probably due to the cell lysis products of starving and dying cells.

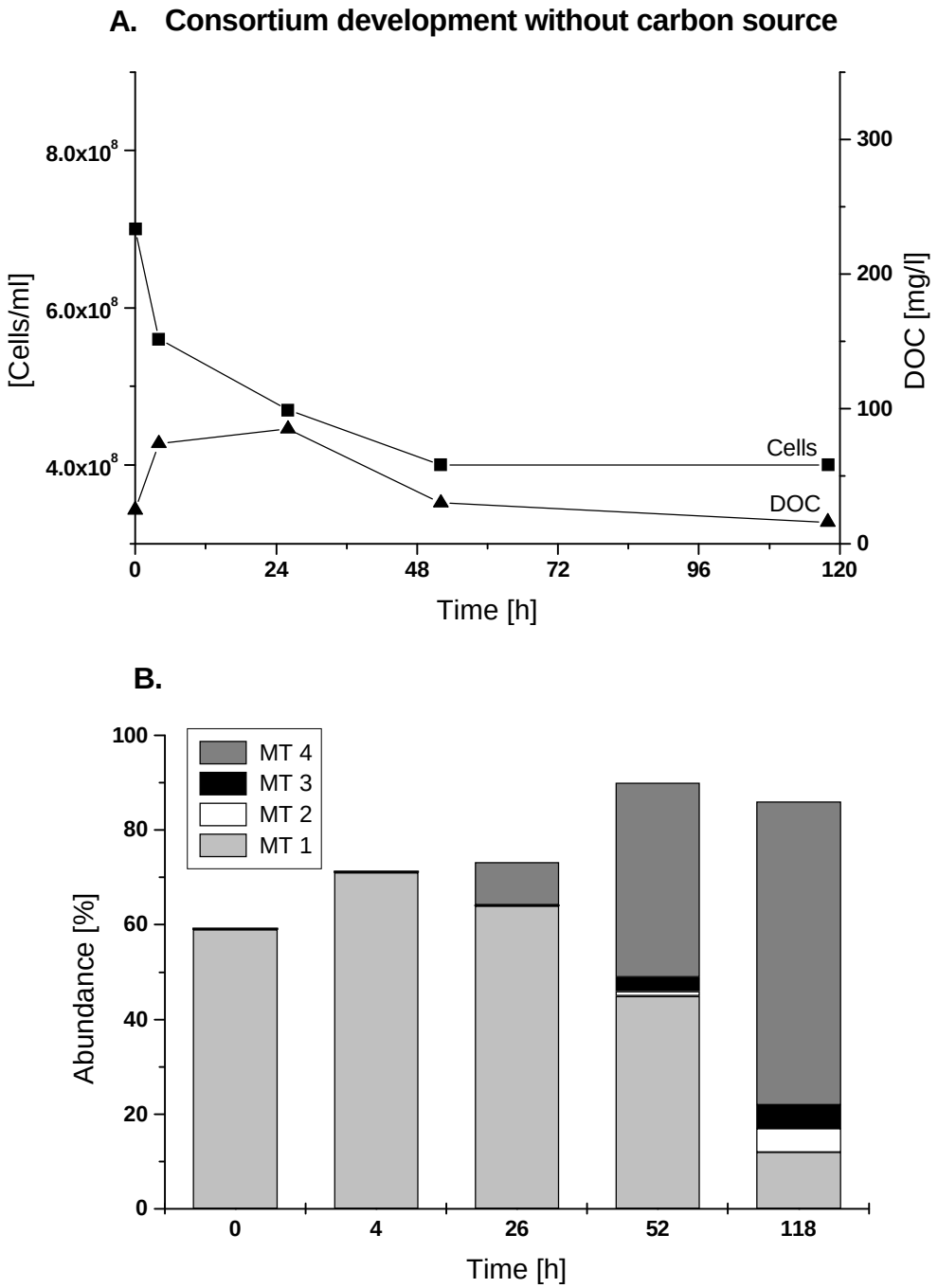

Figure 18: Fed-batch culture of the consortium with minimal medium without carbon source, A. Cell number (■) and measured DOC (▲), B. Abundance of the consortium members.

When feeding was started again after 24 h, a dilution effect was observed, cell number and DOC diminished. The background concentration of 30 mg C l^{-1} was reached. The interesting result of this experiment was the increase of the abundance of *P*. sp. MT 4 based on growth of the strain, as can be seen in Figure 18B and Figure 19. This strain became more than 50% in abundance. In addition, the abundance of consortium member *E. brevis* MT 2 increased from 1% to 10%.

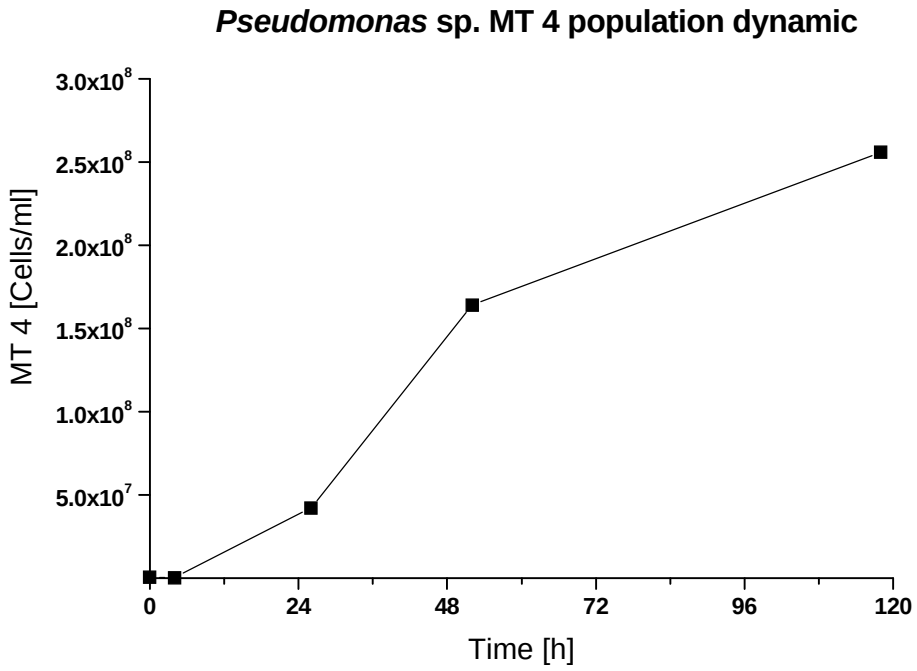

Figure 19: Population dynamic of *P.* sp. MT 4 in fed-batch culture of consortium with minimal medium without carbon source, MT 4 cell number (■)

A possible explanation for the growth of *P.* sp. MT 4 is that this strain (and probably to a lesser degree also *E. brevis* MT 2) was able to survive and utilise cell debris and dying cells. It can not be excluded that all consortium members are able to use cell debris as a carbon source. The observations indicate that *P.* sp. MT 4 and *E. brevis* MT 2 have probably the highest affinity for this kind of carbon source. This observation is relevant, because the increase of *P.* sp. MT 4 after the application of a disturbance might not only be based on a reaction to the disturbance, it is possible that the disturbance resulted in the dying of consortium cells and that *P.* sp. MT 4 utilises this cell debris.

3.3 Mixed substrate utilisation

The 4-CS degrading consortium obtained energy and subsisted on 4-CS, which was converted to CO_2 and H_2O. An alternative, easy to utilise, carbon source may disturb this process dramatically. This issue is interesting, as the quantity and composition of readily available dissolved organic matter is one of the important parameters influencing the biodegradation of pollutants under environmental conditions (Egli, 1995). The influence of a second carbon source upon the structure and function of the 4-CS degrading consortium was studied. In general mixed utilisation studies the addition of sugars (and other substrates like lactate, acetate, butyrate or succinate) to a microbial strain growing on glucose is studied. In the field microorganisms rarely encounter carbon sources like sugars. Therefore were in this thesis alcohols (ethanol, iso-propanol), an amino acid (histidine) and a complex medium ($^1/_{10}$ nutrient broth (NB)) applied as second carbon sources. The amino acid and the alcohols could be easily utilised by some consortium members, are easy to handle and not so much energy can be gained from them as from sugars. Single substrates, not a complex medium, were selected. This is better for metabolic calculations and provides the possibility of labelling the substrate and monitoring its degradation in future experiments. $^1/_{10}$ NB was included because it was shown previously (Frech, 1996), that the substitution of 4-CS by $^1/_{10}$ NB had an influence on the consortium structure.

The optical density, dissolved organic carbon (DOC) and 4-CS concentration were monitored. The DOC concentration measures the sum of the 4-CS and of the second added carbon source concentration. Calculating the difference between the DOC and the 4-CS concentration will reveal whether both carbon sources, or which of the single carbon sources, were degraded. The concentrations of the DOC and 4-CS correspond to each other, with a concentration of 10 mM 4-CS corresponding to a DOC of 720 mgC l^{-1}. The size of the axes of the mixed substrate utilisation graphs were designed accordingly.

3.3.1 Pre-experiments for mixed substrate utilisation studies

The following pre-experiments were performed: first, in batch culture, the metabolic abilities (grown on the selected second carbon sources) of pure cultures of the four consortium strains were analysed (Chapter 3.3.1.1); also, in batch culture, the influence of the simultaneous feeding of 4-CS and α-cyclodextrine or histidine or thymidine on the consortium growth was studied (Chapter 3.3.1.2); in addition, in fed-batch culture, the influence of simultaneous feeding of 4-CS and ethanol or iso-propanol and the influence of $^1/_{10}$ NB as carbon source on the consortium growth was analysed (Chapter 3.3.1.3). Batch and fed-batch culture served as screening tools, as they can be performed faster than the continuous culture.

3.3.1.1 Growth capabilities of pure cultures of consortium strains in batch culture

Growth of pure cultures of the consortium members in batch culture with $^1/_{10}$ NB, ethanol, iso-propanol and histidine was studied (Material and Methods 2.5.3.3).

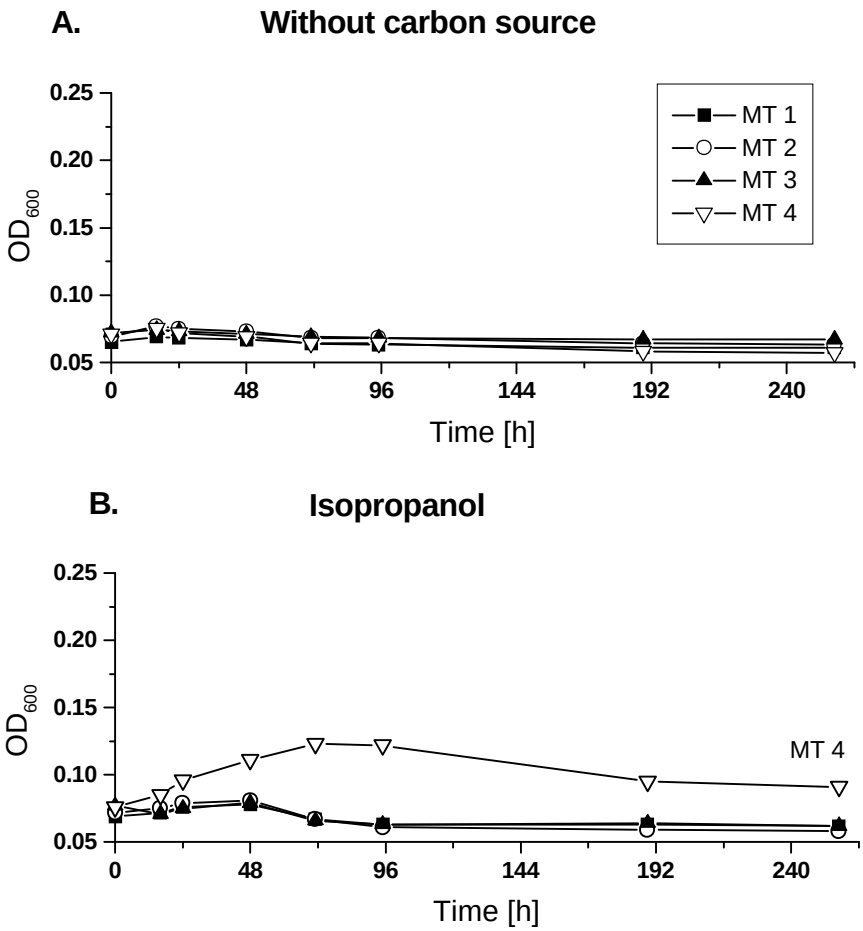

Figure 20: Optical density of pure cultures of the consortium strains maintained in batch culture (MT 1(■), MT 2 (○), MT 3 (▲) and MT 4(▽)) with different carbon sources: A. minimal medium, B. 6 mM iso-propanol;

Only *P.* sp. MT 4 was capable to grow on iso-propanol (see Figure 20B). Figure 21A shows the growth of the consortium members on $^{1}/_{10}$ NB, which can be used by all strains as a carbon source and served as positive control. *E. brevis* MT 2 showed the fastest growth and had the highest yield with on $^{1}/_{10}$ NB. When ethanol was used as carbon source (Figure 21B), only *P.* sp. MT 4 used it as a growth source immediately, and after two days of adaptation, *A. xylosoxidans* MT 3 also grew with this carbon source. Three strains, *P.* sp. MT 1, *P.* sp. MT 4 and *A. xylosoxidans* MT 3 used histidine as a carbon source for growth after an adaptation phase of two days (Figure 21C). *P.* sp. MT 1 grew faster than the other two strains.

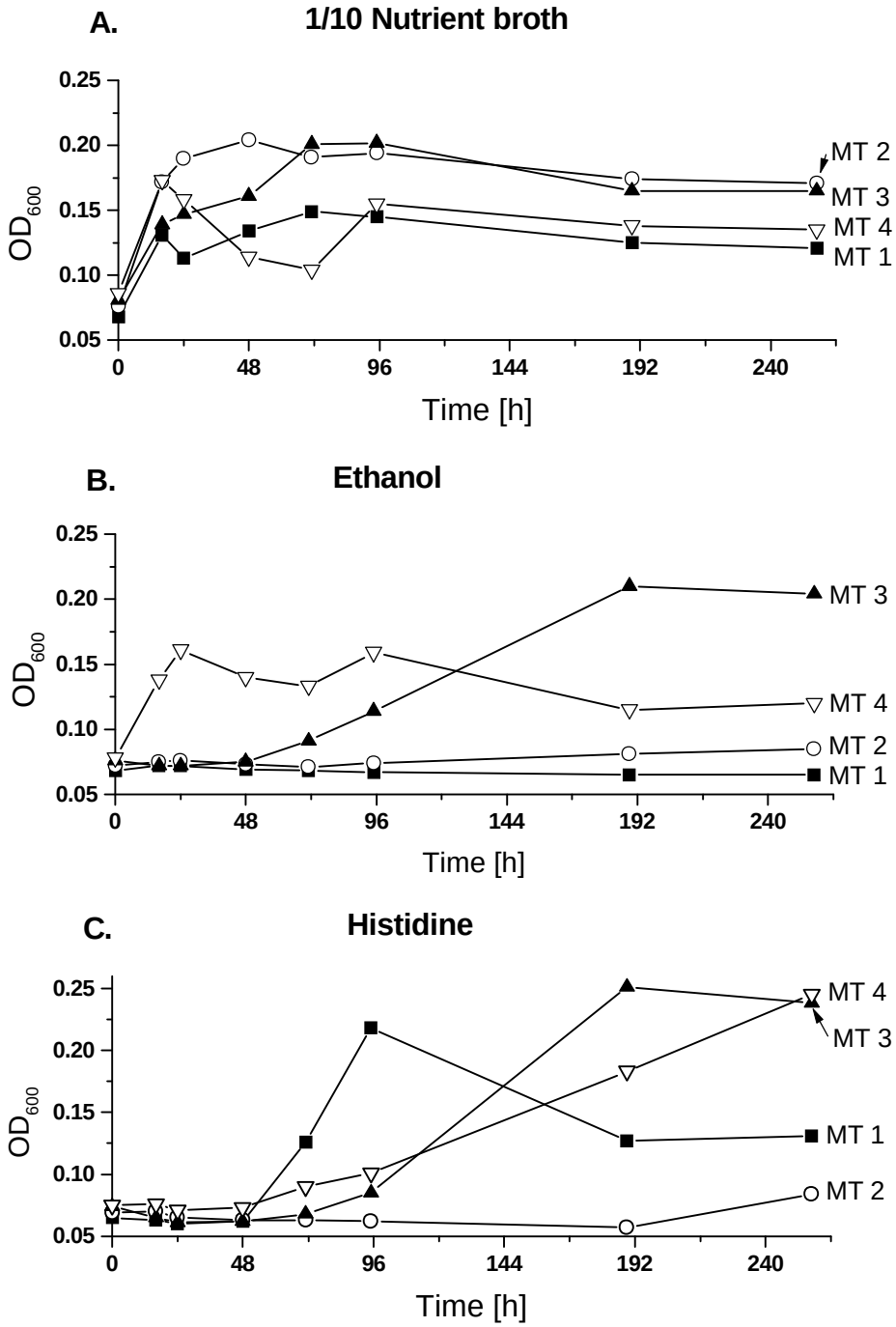

Figure 21: Optical density of pure cultures of the consortium strains maintained in batch culture (MT 1(■), MT 2 (○), MT 3 (▲) and MT 4(▽)) with different carbon sources: A. $^{1}/_{10}$ NB, B. 6 mM ethanol and C. 6 mM histidine;

3.3.1.2 Influence of second carbon source added to consortium in batch culture

Batch culture of the consortium with 4-CS and an added second carbon source (α-cyclodextrine or histidine or thymidine) should give fast preliminary results about suitable second carbon sources (influencing consortium composition). The results of these experiments lead to the selection of histidine as a second carbon source which was then added to the consortium growing in continuous culture on 5 mM 4-CS.

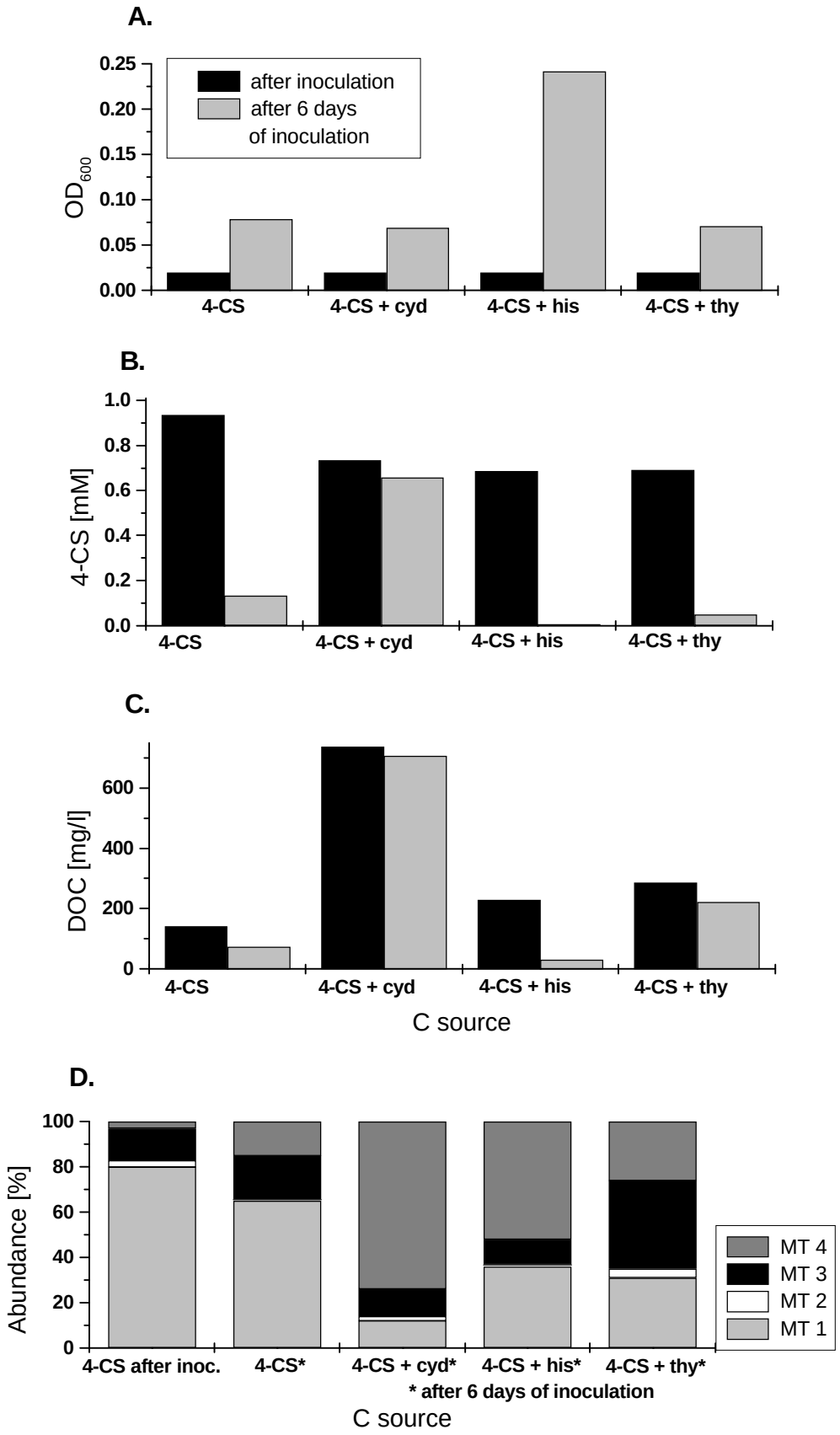

Figure 22: Influence of different carbon sources (4-chlorosalicylate (4-CS), α-cyclodextrine (cyd), histidine (his) and thymidine (thy)) on a mixed culture maintained in batch culture, before and after 6 days of incubation; A. Optical density, B. 4-CS concentration, C. DOC and D. abundance of community members

Batch cultures in 100 ml Erlenmeyer flasks were supplied with 1 mM 4-CS and the second carbon source in equimolar amounts and an inoculum of the consortium (see Materials and Methods 2.5.3.2), except α-cyclodextrine, which was added in a higher concentration.

In the control experiment (only 4-CS as substrate), the expected OD_{600} of 0.08 was reached, and almost all 4-CS was degraded. The consortium structure matched the expected composition (see Chapter 3.2.2).
The concentration of α-cyclodextrine was four times higher than the 4-CS concentration (see Figure 22C). Although the optical density had increased from 0.02 at the beginning to 0.07 after 6 days, only tiny amounts of 4-CS but no cyclodextrine was degraded (Figure 22). The occurrence of metabolites was not monitored. The simultaneous feeding of 4-CS and cyclodextrine in batch culture resulted in no degradation of 4-CS and α-cyclodextrine, and cell death occurred. A shift in consortium composition was observed, and *P.* sp. MT 4 was more than 50% in abundance at the end of the experiment. According to the results of Chapter 3.2.3 did *P.* sp. MT 4 metabolise and grow on the cell debris of the dying cells. In the batch experiment with 4-CS and histidine, the optical density reached 0.23 (see Figure 22A), which was more than double the amount of the control experiment, indicating that the cells could metabolise and grow on both carbon sources. All 4-CS was degraded and the final DOC concentration was 30 (mg C) l^{-1} (the background value), showing both substrates to be degraded. The community composition had changed to 50% *P.* sp. MT 1 and *E. brevis* MT 2 and *A. xylosoxidans* MT 3 and 50% *P.* sp. MT 4 (Figure 22D). The absolute abundance was calculated from the optical density using the equation from Chapter 2.6.2.

The plot of the absolute abundance (see Figure 23) showed that the concentration of *P.* sp. MT 1 cells at the end of the histidine addition experiment was approx. 2.5×10^8 cells ml^{-1}, which is higher than the concentration of *P.* sp. MT 1 cells (approximately 1×10^8 cells ml^{-1}) at the end of the experiment with only 4-CS feeding. The ability of *P.* sp. MT 1 to grow on histidine fastest was shown in the previous chapter (see Figure 22) Additionally did *P.* sp. MT 4 grow on histidine. The OD_{600}, indicating the cell concentration, was high, as the degradation of 1 mM 4-CS resulted in an optical density of 0.08, double the amount of carbon source that should result in the double optical density, 0.16. But the observed OD_{600} was 0.24. It is possible that a different carbon source might lead to higher cell numbers, as histidine might have a higher yield of biomass production (see Figure 22). The simultaneous feeding of 4-CS and histidine in batch culture resulted in the simultaneous or diauxic degradation of histidine and 4-CS. *P.* sp. MT 1 and *P.* sp. MT 4 did use histidine for growth, this was reflected in the doubling of the absolute abundance for *P.* sp. MT 1 and in the gain of 50% in relative abundance for *P.* sp. MT 4.

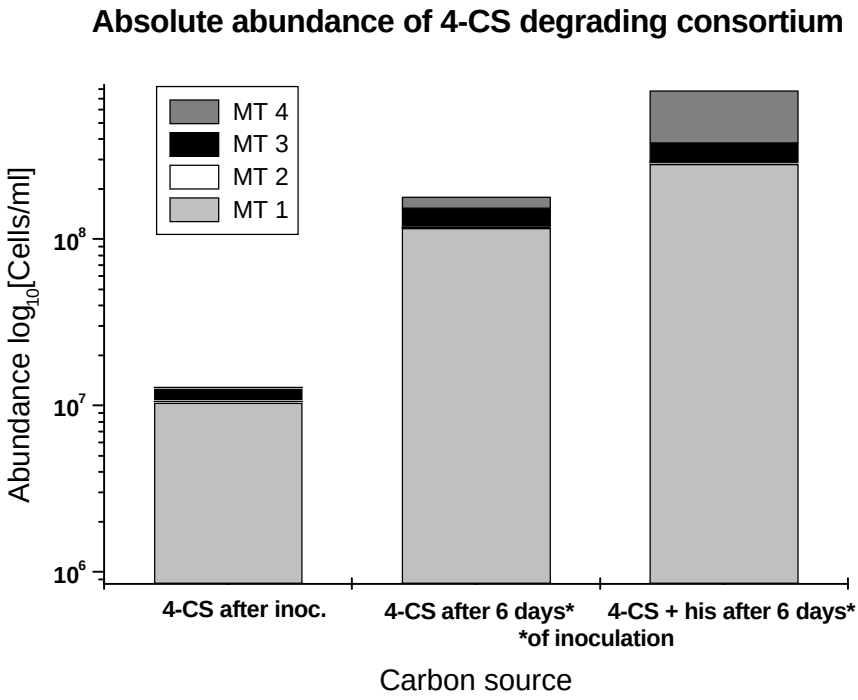

Figure 23: Consortium abundance after inoculation and 6 days of inoculation with 4-CS and with 4-CS and histidine in batch culture, absolute abundance of consortium members

The additional feeding of thymidine led to an increase of the OD_{600} to 0.08 (Figure 22). The value that would be expected for the degradation of 1 mM 4-CS. 4-CS was degraded, but thymidine was not degraded, as indicated by the DOC. The consortium composition changed in that *P.* sp. MT 1 lost in abundance (from 80% to 30%) while *A. xylosoxidans* MT 3 gained in abundance (from 15% to 40%). *P.* sp. MT 4 also increased in abundance. The simultaneous feeding of 4-CS and thymidine in batch culture resulted in the degradation of 4-CS, but no degradation (or taking up) of thymidine. The consortium composition to a lower abundance of *P.* sp. MT 1 and higher abundance of *A. xylosoxidans* MT 3 and *P.* sp. MT 4, perhaps due to inhibition of *P.* sp. MT 1 by thymidine.

As neither α-cyclodextrine nor thymidine were degraded by the consortium, only histidine was selected as suitable second carbon source, and a continuous culture experiment with additional histidine feeding was performed.

3.3.1.3 Mixed substrate utilisation and substitution of 4-CS by $^1/_{10}$ NB studied in fed-batch culture

Fed-batch culture provides a fast, technically easy, way to study the influence of the feeding of two carbon sources upon the consortium. In batch culture, the conditions are always changing, and in fed-batch culture an almost steady-state can be reached without the effort of setting up a continuous culture. The feedstock for the fed-batch culture contained 5 mM 4-CS

and, in addition, ethanol or iso-propanol, respectively. In a further experiment, the consortium was fed with $^1/_{10}$ NB as only carbon source. An experiment, in which 4-CS was exchanged by $^1/_{10}$ NB, was performed before by Frech (1996). The repeat in this thesis should show whether the consortium would show the same reaction as before to validate the consortium performance. The fed-batch culture was performed as described in Material and Methods 2.5.4.

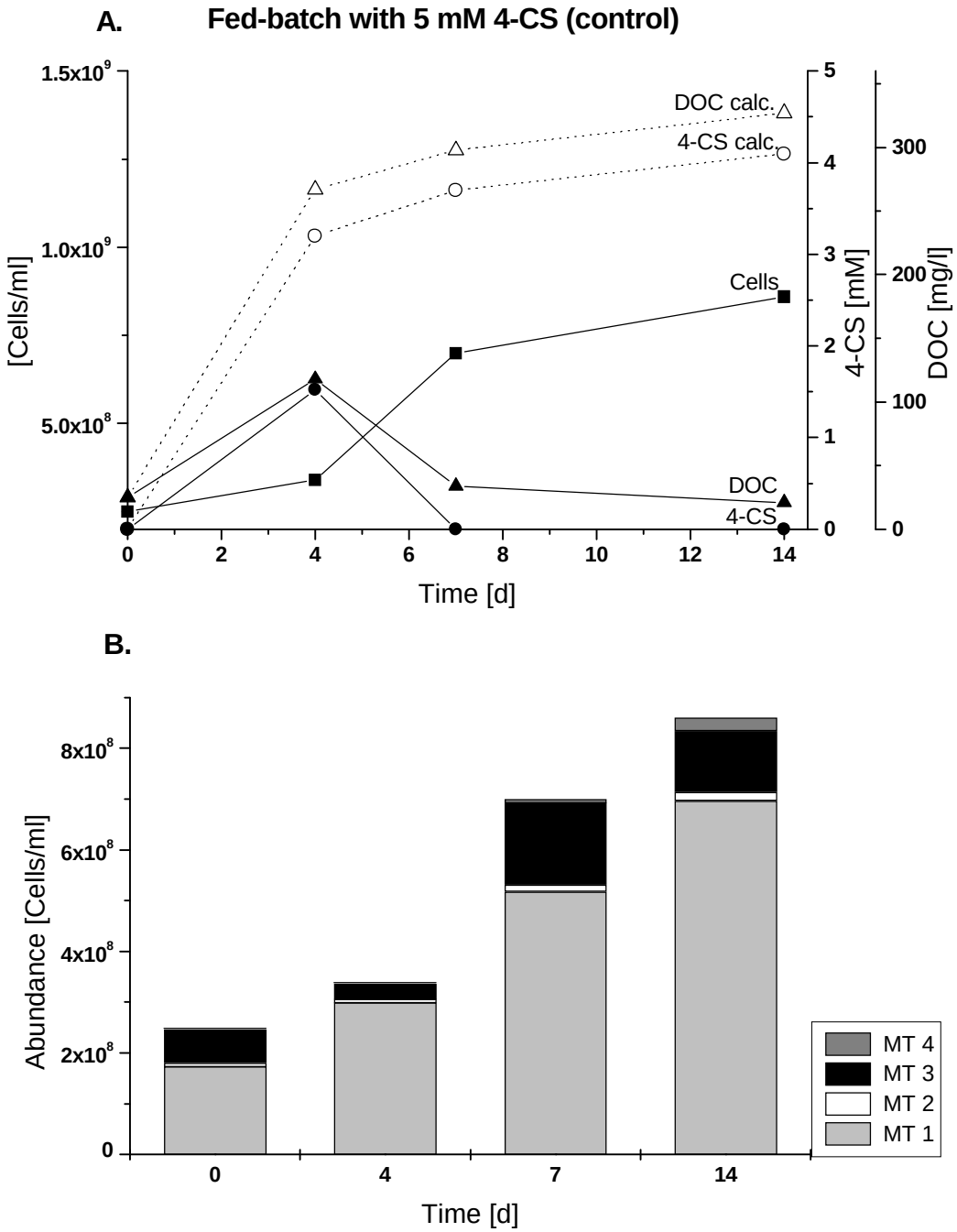

Figure 24: Fed-batch of consortium with 5 mM 4-CS in medium reservoir, flow rate 1 ml h^{-1} until day 3; then 0.5 ml h^{-1}, A. Cell number (■), 4-CS calculated, without degradation (○) and measured (●), DOC calculated, without degradation (△) and measured (▲); B. Abundance of consortium members

The substrate addition experiments match up to the control experiment shown above. Consortium growth with 5 mM 4-CS at two different feeding rates is shown in Figure 24. The flow rate of 1 ml h^{-1}, which was applied during the first 4 days, was to high, 4-CS accumulated in the medium. The cells were still able to survive, as the 4-CS concentration did not exceed 2 mM. About half of the 4-CS was degraded and therefore the cell number increased. The biomass consisted only of consortium cells, no contamination (see relative abundance (Figure 25)) occurred. The consortium structure at the end of the experiment was MT 1 = 81%, MT 2 = 2%, MT 3 = 14%, MT 4 = 3%, this matches the values expected for undisturbed consortium growth (see Chapter 3.2.2).

After 4 days the flow rate was reduced to 0.5 ml h^{-1} and not only the inflowing 4-CS was degraded, but also the concentration in the reactor vessel decreased and the cell number increased rapidly. After 7 days all 4-CS was degraded and a steady state was reached. The amount of carbon source (4-CS) controlled the growth of the cells and the corresponding amount of biomass was produced. The cell number should stay constant, as the cells are growing at the rate that 4-CS is fed thus increasing the volume. This graph shows the development of the undisturbed consortium. In this experiment the DOC equals the amount of 4-CS, because only one carbon source was used.

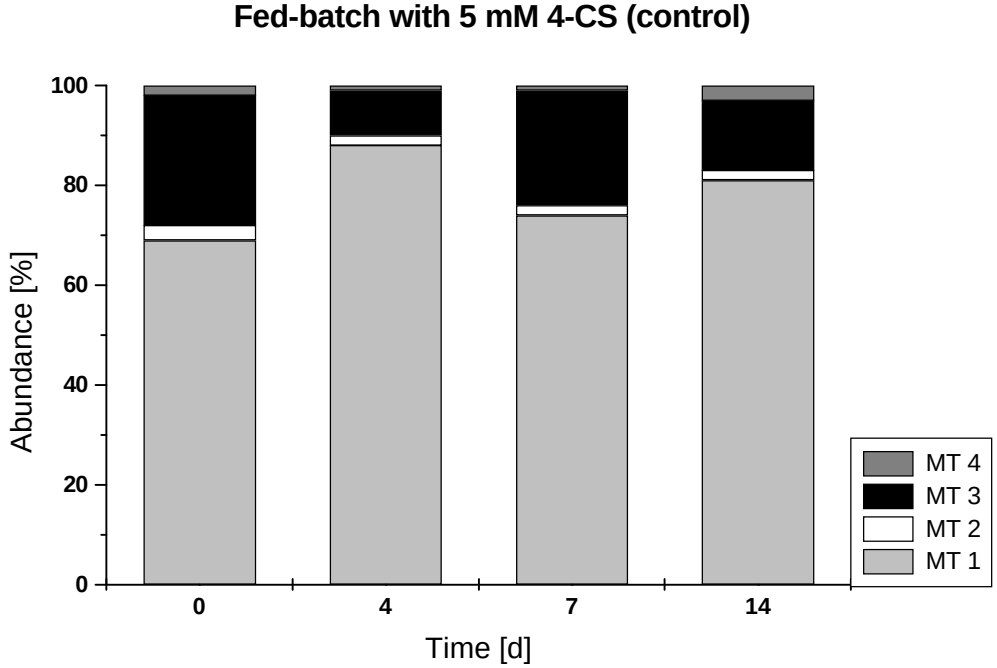

Figure 25: Fed-batch culture of 4-CS degrading consortium with 5 mM 4-CS as feedstock concentration; Relative abundance of consortium members.

The additional ethanol and iso-propanol feeding experiments were treated in the same way. First the higher flow rate of 1 ml h^{-1} for 3 days was applied, followed by a rate adjustment to

0.5 ml h^{-1}, which created a variation within the experiment. In addition to the DOC, the 4-CS concentration was measured (Materials and Methods 2.8). The theoretical concentration of the carbon sources without degradation was displayed to show whether degradation had occurred.

The fed-batch experiment with 5 mM 4-CS and 15 mM ethanol as carbon sources is shown in Figure 26. The DOC content in the feedstock was comprised of 5 mM 4-CS and 15 mM ethanol. Only half of the added carbon mixture was degraded, and the rest accumulated in the reaction vessel. The difference between the theoretical development of the DOC and the 4-CS concentration shows that although ethanol was degraded, 4-CS was not. The cell number increased until day 4 and this was due to the increase of consortium member *P*. sp. MT 4 (Figure 26B). A pure culture of *P*. sp. MT 4 grew on ethanol without a lag phase and faster than *A. xylosoxidans* MT 3 (see Chapter 3.3.1.1, Figure 21B). *A. xylosoxidans* MT 3 was the only other consortium strain that could use ethanol as a carbon source (Figure 21B). *P*. sp. MT 4 reached 50% in abundance (see Figure 27). When the flow rate was reduced, the total cell number decreased.

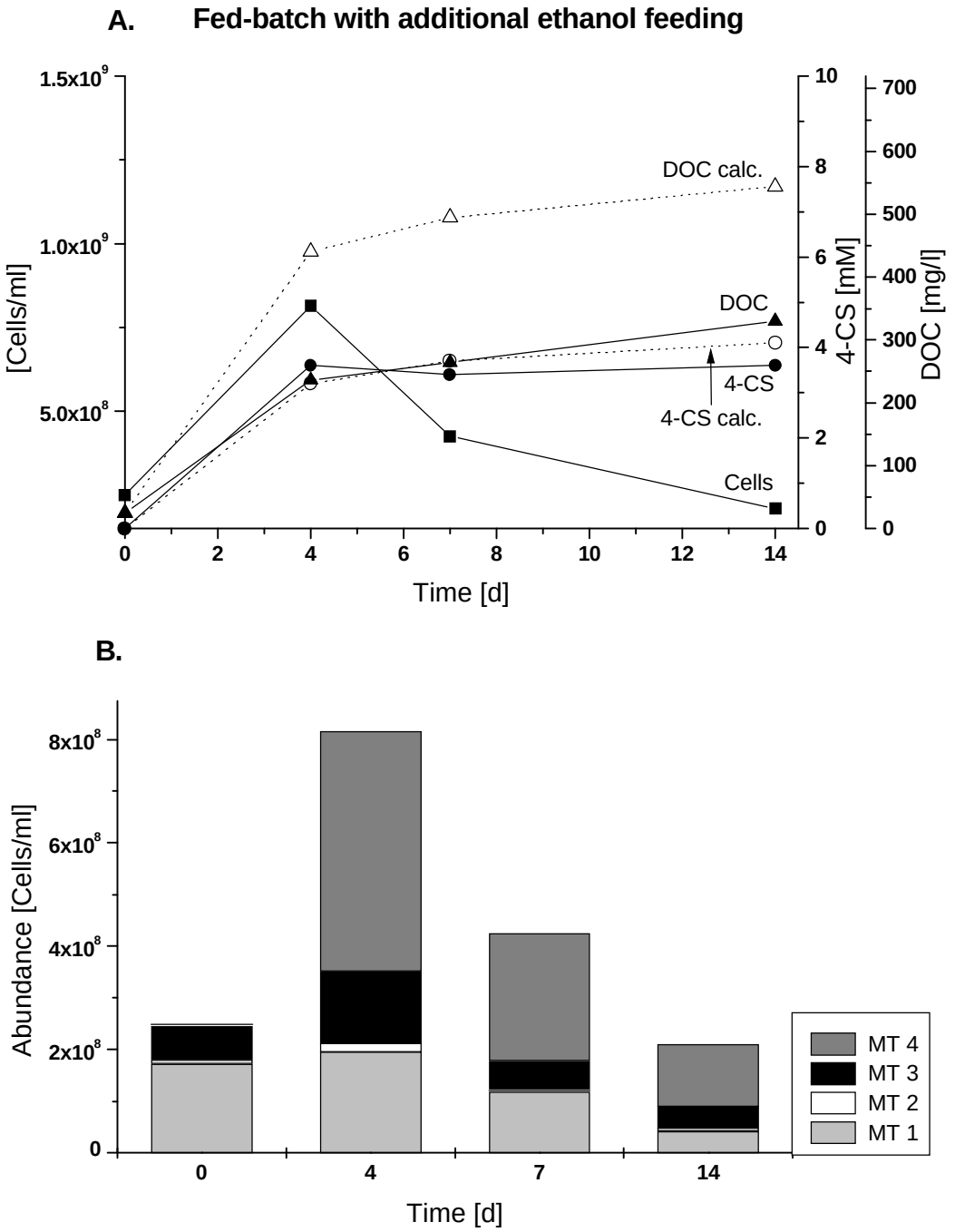

Figure 26: Fed-batch of consortium with 5 mM 4-CS and 15 mM ethanol in medium reservoir, A. Cell number (■), 4-CS calculated, without degradation (○) and measured (●), DOC calculated, without degradation (△) and measured (▲); B. Abundance of consortium members

Although the overall cell number decreased, the consortium structure during this dilution phase did not vary much, and the structure was MT 1 = 24%, MT 2 = 2%, MT 3 = 17%, MT 4 = 57%. Of the two fed carbon sources, only ethanol was degraded, leaving 4-CS accumulated in the medium. When the 4-CS concentration exceeded 2 mM (see Figure 26A), dilution of the cells in fed-batch culture occurred, probably due to the inhibition of strain *P.* sp. MT 1 and the other consortium members, because in previous experiments, reduced growth rates and

yields of *P*. sp. MT 1 and the consortium at 4-CS concentrations higher than 2 mM were observed (shown in Chapter 3.1.3 and 3.1.5). It is possible that *P*. sp. MT 4 was still able to degrade ethanol because ethanol did not accumulate in the medium. But the cell number of *P*. sp. MT 4 decreased also. The reduction of the flow rate reduced the ethanol influx, and the lower concentration could only support the growth of a lower *P*. sp. MT 4 cell number.

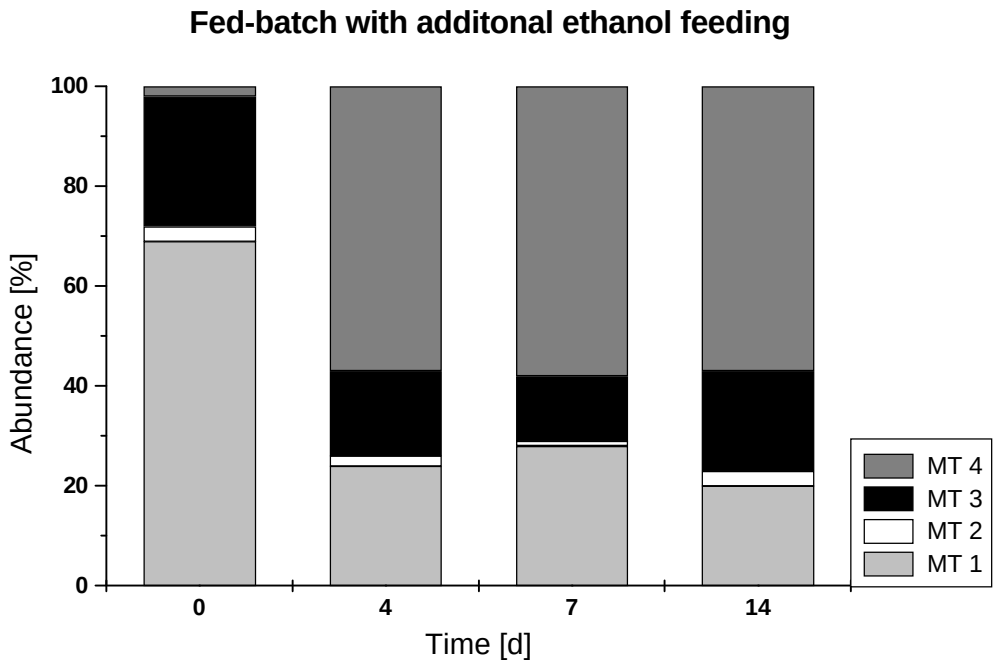

Figure 27: Fed-batch culture of 4-CS degrading consortium with 5 mM 4-CS and 15 mM ethanol feedstock concentration; Relative abundance of consortium members.

A fed-batch experiment with additional iso-propanol feeding exhibited a similar development (see Appendix 6.2.1, Figure 55). The alcohol was degraded, but 4-CS accumulated. The consortium structure changed, as *P*. sp. MT 4 increased to about 30% - 50% in abundance. The consortium structure at the end of the experiment was MT 1 = 34%, MT 2 = 1%, MT 3 = 11%, MT 4 = 54%. When the flow rate was reduced the cell number decreased, and an increase of *P*. sp. MT 1 from the start of the experiment until day 4 was observed. A minor increase of the total cell number at the end of the experiment was observed.

In a different series of fed-batch experiments, 4-CS was exchanged by $^{1}/_{10}$ NB as a carbon source. This experiment belonged to a different set of fed-batch experiments. The undisturbed control was introduced in Chapter 3.2.3, Figure 17 and Figure 18. The consortium structure at the beginning of the experiment was influenced by the existence of a contaminant. The carbon source was fed at a rate of 1.5 ml h^{-1} for the first 4 h, then the feeding was stopped, resulting in a batch growth phase of 20 h. After 1 d the feeding rate was reduced to 1 ml h^{-1}.

The development of the consortium grown in fed-batch culture with $^{1}/_{10}$ NB as a carbon source is shown in Figure 28. After 4 h, a rapid increase in the DOC can be observed (Figure 28A). The DOC concentration was higher than expected, probably due to the initial pump adjustment allowing extra medium to be fed. In the first 4 h, an unexpected decrease in the cell number was also observed, most likely due to a lag phase, as the cells had to adapt to the new carbon source. The cell number decrease could have been caused by the additional volume increase resulting from the pump adjustment. After the batch phase, at 24 h the carbon source was degraded and *E. brevis* MT 2 had already increased in abundance. It is highly probable that *E. brevis* MT 2 has the highest substrate affinity to NB, although all strains were able to use NB as a carbon source (see Chapter 3.3.1.1). Obviously, *E. brevis* MT 2 outcompeted the other strains. When feeding was started again, the cell number increased, and the majority of the available carbon was degraded and the abundance of *E. brevis* MT 2 increased further. At the end of the experiment, a steady state was reached.

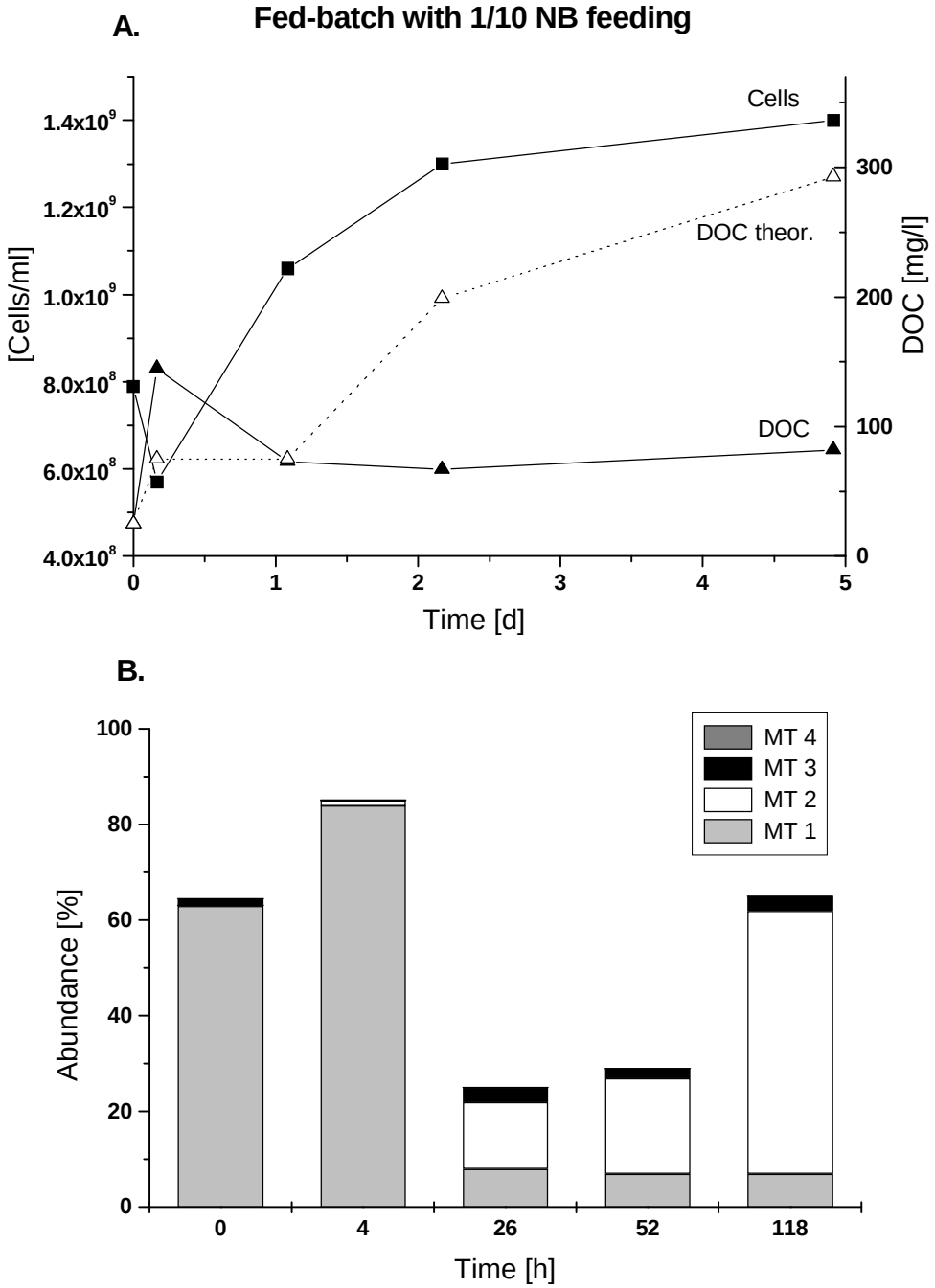

Figure 28: Fed-batch culture of the consortium with $^{1}/_{10}$ NB (nutrient broth), A. Cell number (■) and measured DOC (▲), calculated DOC (△) without degradation, B. Abundance of the consortium members.

The majority of the available carbon was degraded, and the cells grew as fast as the carbon was fed. Feeding of minimal medium resulted in a DOC of 30 mg C l^{-1} (see Chapter 3.2.3), but the background DOC, which was observed in the $^{1}/_{10}$ NB feeding experiment, was higher (80 mg C l^{-1}). As NB is a complex medium, it is possible that it was not completely metabolised. Another possibility is that a metabolite of the NB degradation was produced and accumulated. The increase of *E. brevis* MT 2 (Figure 28B) was expected, as this occurred in Frech´s work (Frech, 1996). This experiment showed that even with a unfavourable

community composition at the beginning, the effect of the influence of the carbon source exchange from 4-CS to $^{1}/_{10}$ NB resulted in the increase in the abundance of *E. brevis* MT 2. With a substance that can be degraded faster than 4-CS, steady state conditions were achieved in fed-batch culture.

In summary:

- The simultaneous feeding of 4-CS and ethanol and of 4-CS and iso-propanol in fed-batch culture resulted in the degradation of the alcohols. 4-CS was not degraded. A shift in the consortium composition was observed, and *P*. sp. MT 4 increased to 50% in relative abundance. The accumulation of 4-CS to more than 2 mM inhibited the consortium members, which led to the dilution of cells.
- The exchange of 4-CS by $^{1}/_{10}$ NB resulted in the expected consortium shift, and *E. brevis* MT 2 increased in abundance.

Ethanol and iso-propanol were selected as carbon sources, which had a negative effect upon the consortium function. Continuous culture experiments were performed with these second carbon sources. Like Frech's previously observed consortium shift, a shift was also here observed, with an increase in abundance of *E. brevis* MT 2, due to feeding of $^{1}/_{10}$ NB. The feeding of this substrate had an influence upon the abundance of the consortium member *E. brevis* MT 2 and not on *P*. sp. MT 4, like the other carbon sources. These observations resulted in the selection of $^{1}/_{10}$ NB as second carbon source for one further experiment. The influence of $^{1}/_{10}$ NB as a second carbon source and not as a single carbon source was studied in continuous culture.

3.3.2 Mixed substrate utilisation in continuous culture

The preliminary experiments gave insights into how the addition of different carbon sources affected consortium structure and composition. Continuous culture experiments with the consortium fed with 5 mM 4-CS and one additional carbon source were performed as described in Materials and Methods 2.5.5.4. As a prerequisite, an undisturbed steady state of the consortium growing on 5 mM 4-CS in continuous culture exists. This undisturbed steady state was disturbed by the start of the feeding of the second carbon source, which will probably result in a new steady state. When the additional feeding of the second carbon source is stopped, it can then be observed whether the consortium returns to the pre-disturbed state. The time for the continuous culture experiments is longer than the time for batch or fed-batch experiments, which were limited by the amount of carbon source or the volume of the reaction vessel. Six 500 ml chemostats were started and maintained in parallel. All of them were fed with 5 mM 4-CS as a sole source of carbon and energy. When the disturbance was applied, the medium reservoirs of the single experiments were substituted by new medium reservoirs, consisting of the minimal medium with 4-CS and the second carbon source. As control, one chemostat was continuously fed with only 4-CS.

The following carbon sources were selected and the following mentioned results of the experiments were expected. The additional feeding of

- ethanol and iso-propanol should lead to a degradation of the alcohol by consortium member *P*. sp. MT 4, but 4-CS should not be degraded (observed in Chapter 3.3.1.3); this should result in a breakdown of the system.
- histidine should lead to simultaneous degradation of both carbon sources, by *P*. sp. MT 4 and the consortium (observed in Chapter 3.3.1.2).
- $^{1}/_{10}$ NB should lead to a degradation of the NB by *E. brevis* MT 2 (Chapter 3.2.3), but it is unknown if 4-CS will be degraded simultaneously.

The continuous culture of the consortium fed with 4-CS and ethanol is shown in Figure 29. The undisturbed control is shown in Chapter 3.2.1, Figure 14 and Figure 15. A pre-run phase of 7 days with 5 mM 4-CS feeding was sufficient to reach steady state conditions, and then additional ethanol feeding was applied for 12 days. The additional feeding of ethanol led to an increase of the optical density (see Figure 29A). The increase in cell concentration was based on the growth of *P*. sp. MT 4, this can be seen in the relative abundance plot (Figure 30) and in Figure 29B. *P*. sp. MT 4 was the strain which was able to degrade ethanol instantaneously (refer to Chapter 3.3.1.1). Until day 10, both carbon sources were degraded in parallel. From day 10 on, the degradation of 4-CS stopped and 4-CS accumulated in the reaction vessel. *P*. sp. MT 1 ceased growing, and the optical density and cell number diminished. No accumulation of protoanemonin or 4-chlorocatechol was observed (data not shown). The cell number of *P*. sp. MT 4 remained unchanged as long as the ethanol was supplied. At day 14, the concentration of 4-CS overcame the critical concentration of 2 mM in the reaction vessel. Now the consortium cells were inhibited by the 4-CS concentration. The *P*. sp. MT 1 and *A. xylosoxidans* MT 3 cell number diminished, and the cells were washed out. At this point, the DOC measurement was higher than the 4-CS measurement. This was probably due to the accumulation of ethanol in addition to 4-CS. After 19 days, when the additional feeding of ethanol was stopped, the other consortium members and *P*. sp. MT 4 were washed out (see Figure 29B). The system was too much handicapped to recover. The washout continued until the concentration of 4-CS was the same as the feedstock concentration.

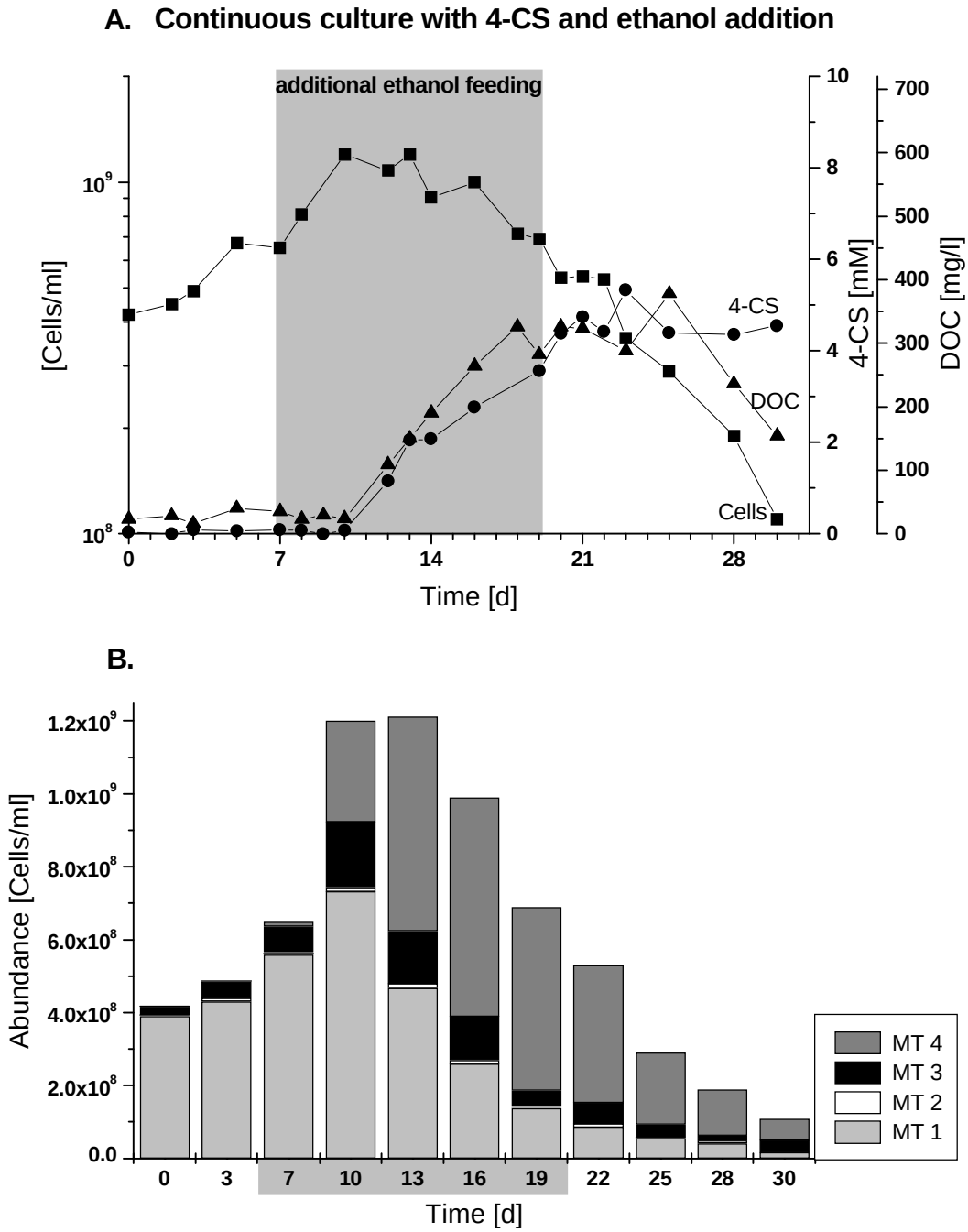

Figure 29: Consortium disturbed by additional feeding of 15 mM ethanol A. Cell number (■), 4-CS concentration (●) and DOC (▲), B. absolute abundance of consortium members

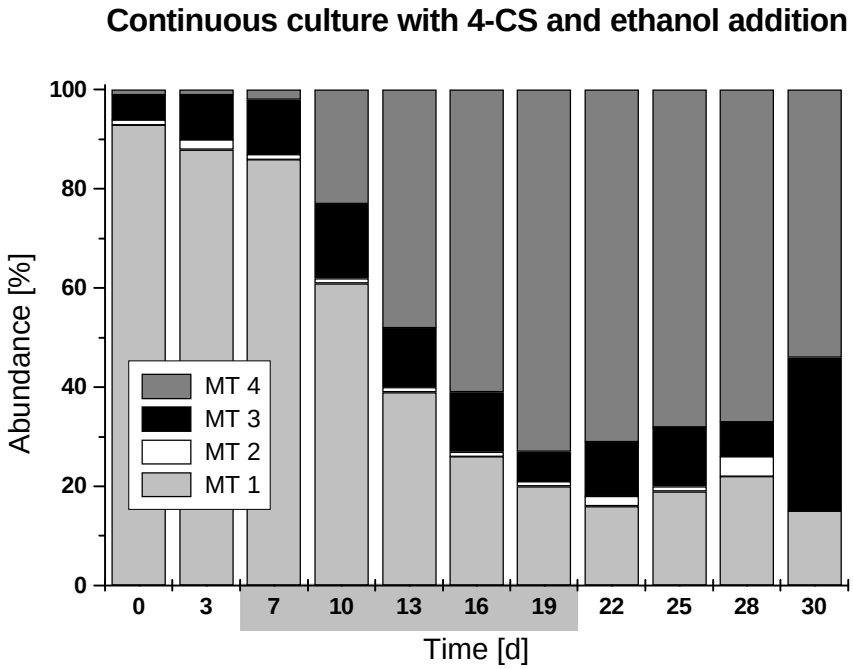

Figure 30: Consortium disturbed by additional feeding of 15 mM ethanol; relative abundance of consortium members

A similar result was found when iso-propanol was fed (Appendix 6.2.2, Figure 57). The only difference was that the whole process was slower. After the start of the additional feeding of iso-propanol, the optical density increased. The abundance of consortium member *P*. sp. MT 4 increased to more than 50% in abundance. After four days, 4-CS accumulated in the reaction vessel, up to 2 mM at day 21. When the additional feeding of iso-propanol was stopped, the system was not able to recover and a washout of the cells occurred. The accumulation of 4-CS continued. It seems that the washout of the cells started when the additional feeding was stopped (at day 21), but in reality, the process started earlier, which is indicated by the accumulation of 4-CS (after day 13).

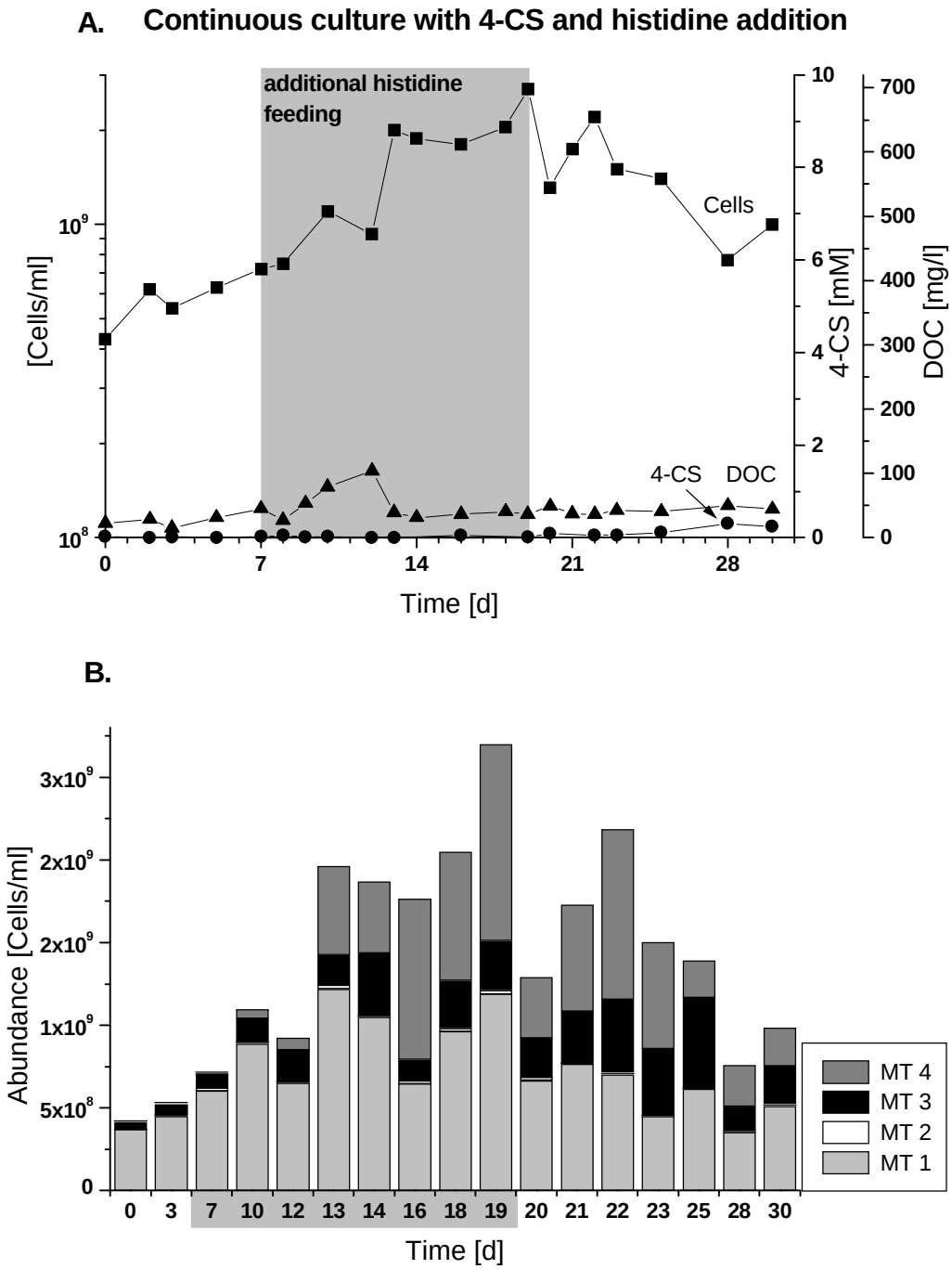

Figure 31: Consortium disturbed by additional feeding of 5 mM histidine A. Cell number (■), 4-CS concentration (●)and DOC (▲), B. absolute abundance of consortium members

The continuous culture of the consortium fed with 4-CS and histidine is shown in Figure 31. A pre-run phase of 7 days with 5 mM 4-CS feeding was sufficient to reach steady state conditions. Then additional histidine feeding was applied for 12 days. The undisturbed control is shown in Chapter 3.2.1, Figure 14 and Figure 15. After 7 days the additional feeding of histidine was started, which resulted in an increase in optical density (refer to Figure 31A). The increase in cell concentration was mainly based on the increase of *P.* sp. MT 4, as can be seen in the plot of the relative abundance (Figure 32).

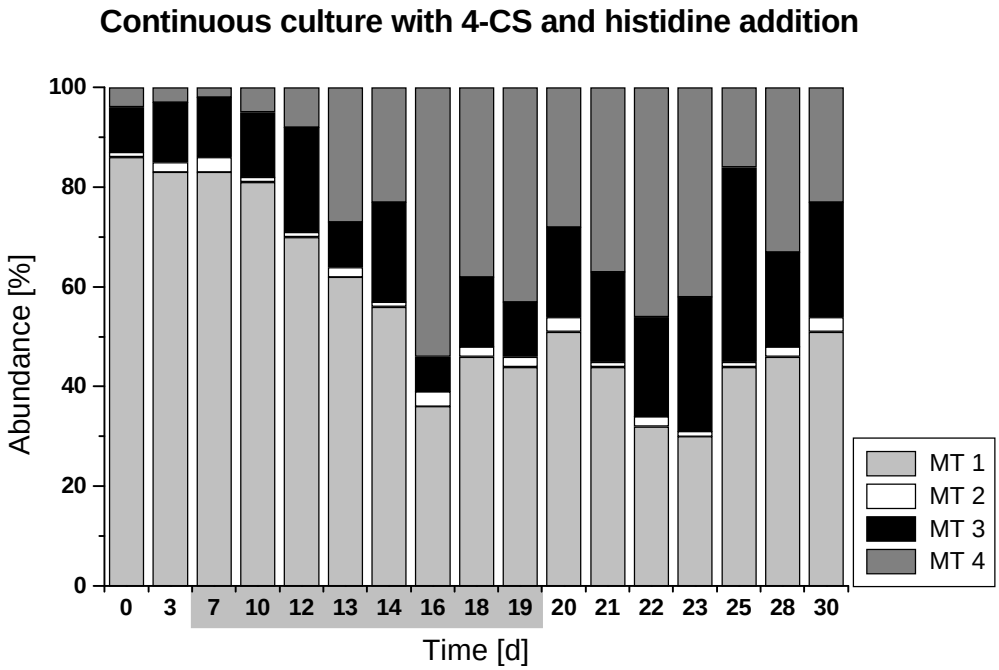

Figure 32: Consortium disturbed by additional feeding of 5 mM histidine; relative abundance of consortium members;

Between day 7 and day 12 the DOC in the reaction vessel increased, most likely due to the accumulation of histidine. (It was not a metabolite of the 4-CS degradation pathway, as no accumulation of catechol, *cis*-dienelactone and protoanemonin was observed). This was probably an adaptation phase of the consortium. From the single substrate addition experiments (Chapter 3.3.1.1), it is known that the strains *P.* sp. MT 1, *A. xylosoxidans* MT 3 and *P.* sp. MT 4 need an adaptation phase of about 4 days after which they can degrade histidine. On day 13, the abundance of *P.* sp. MT 4 had increased (see Figure 31B). Then the optical density increased, the DOC decreased and both carbon sources were degraded in parallel. Between day 14 and day 19, the abundance of *P.* sp. MT 4 was continuously 50%, indicating that *P.* sp. MT 4 degraded histidine. *P.* sp. MT 1 was also able to degrade histidine. The abundance of *P.* sp. MT 1 during the additional feeding of histidine was higher (approximately 1×10^9 cells ml^{-1}) compared to the cell numbers of *P.* sp. MT 1 before and after the disturbance (approximately 5×10^8 cells ml^{-1}) (see Figure 31B). When the additional feeding of histidine was stopped, optical density and consortium composition returned to their pre-disturbed state. Between day 20 and 25, the cell number of *A. xylosoxidans* MT 3 and *P.* sp. MT 4 increased. A similar reaction was found during the early exponential growth in batch culture (Chapter 3.1.1 and Figure 9), which shows that the consortium reacts to the omission of histidine feeding with growth of these two consortium members. *A. xylosoxidans* MT 3 had a necessary role in the development of the consortium composition (see Chapter 4.2.3). 4-CS was continuously degraded, and no system breakdown occurred.

The metabolites protoanemonin and *cis*-dienelactone were monitored in the undisturbed experiment (Chapter 3.2.1, Figure 14), and in the additional ethanol and histidine feeding experiments (data not shown). The concentration of protoanemonin was never higher than 5 µM and of *cis*-dienelactone was never higher than 0.1 mM. This was not enough to cause a system's breakdown.

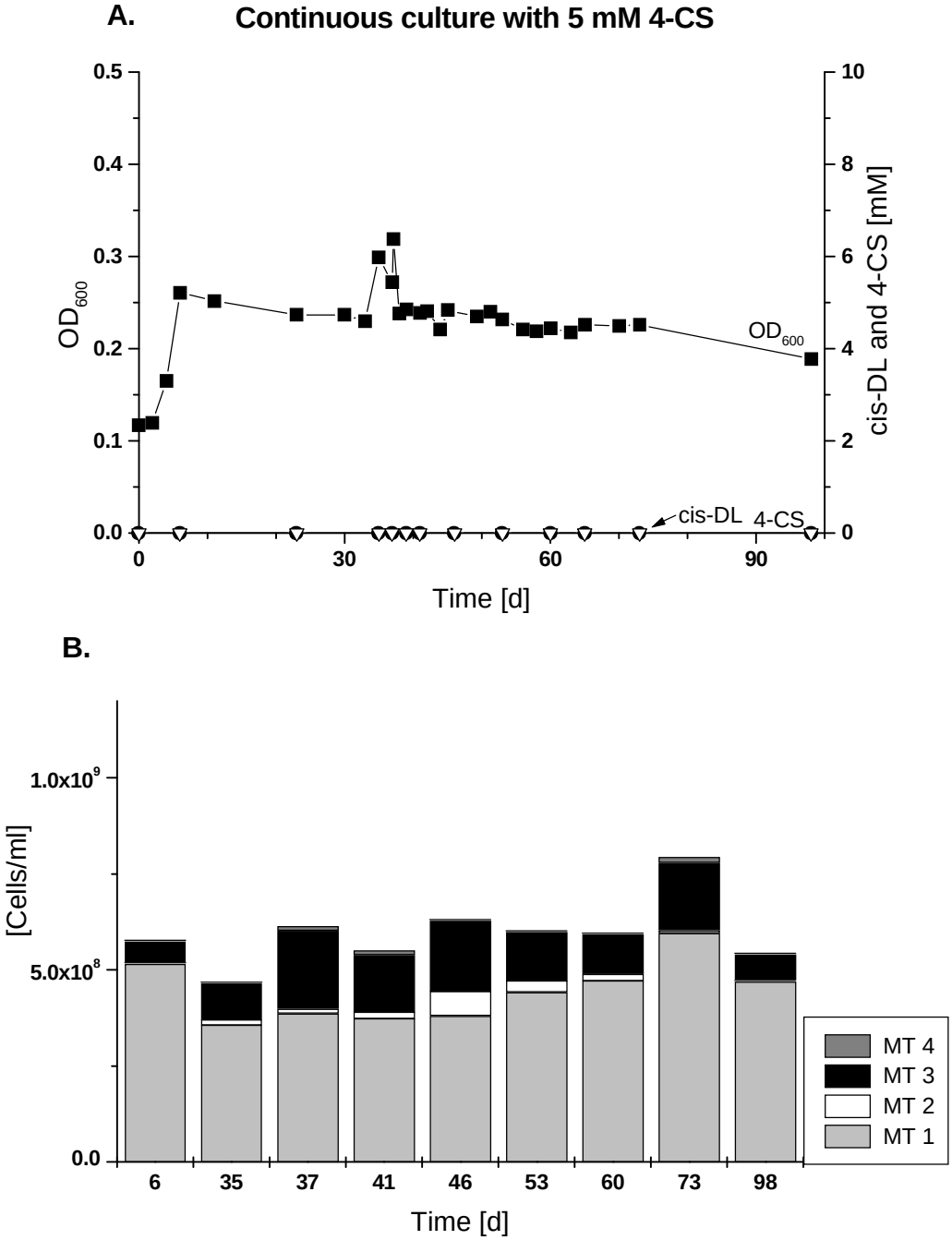

Figure 33: Continuous culture of consortium with 5 mM 4-CS, undisturbed control;
A. OD_{600} (■), 4-CS (●) and metabolite *cis*-dienelactone (▽) concentration,
B. absolute abundance

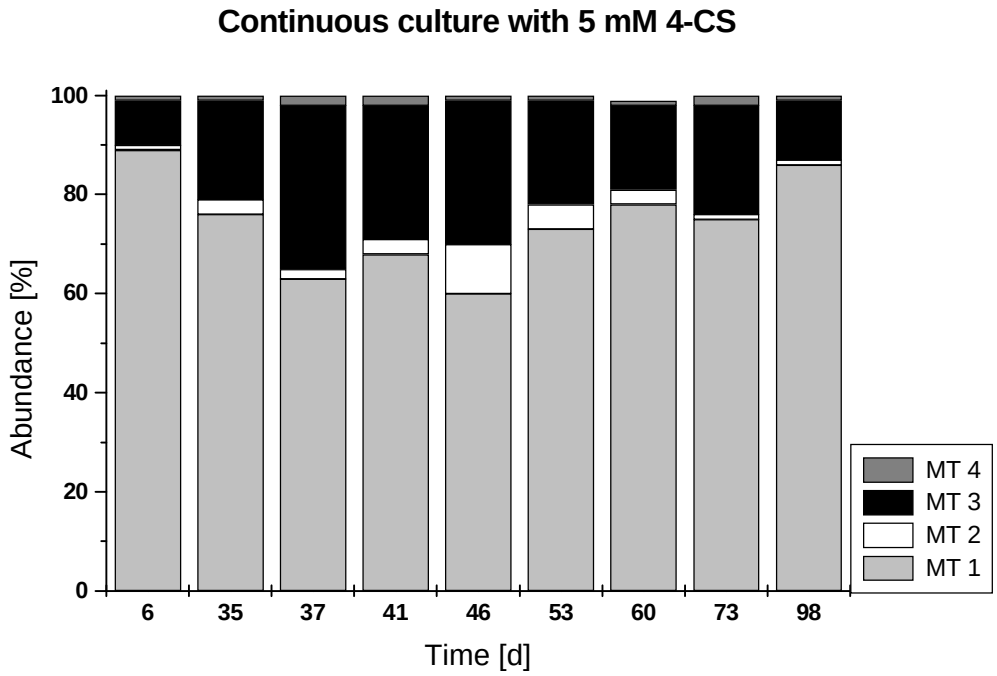

Figure 34: Continuous culture of consortium with 5 mM 4-chlorosalicylate, undisturbed control; relative abundance of consortium members;

The continuous culture of the consortium fed with 4-CS and $^{1}/_{10}$ NB is plotted in Figure 35. This experiment belonged to a different set of continuous cultures. Therefore the experiment has to be related to a different control experiment. The undisturbed control is shown in Figure 33. Complete degradation of 4-CS during the whole experiment and no accumulation of *cis*-dienelactone was observed. Only minor insignificant shifts in the OD_{600} and the consortium structure were detected. On day 32-35 a higher optical density was observed, which was due to additional medium influx created by the application of disturbances in the other chemostats.

The pre-run time before additional feeding of $^{1}/_{10}$ NB was 37 days, and this time was sufficient for three volume exchange ratios to ensure that the steady state conditions were reached. The additional feeding of $^{1}/_{10}$ NB was applied for 27 days, which was sufficient for another three volume exchange ratios. The additional feeding of $^{1}/_{10}$ NB led to an increase of the cell number, which was based on the increase of *E. brevis* MT 2 between day 37 and day 53 (see Figure 35B). Pictures of the indirect immunofluorescence observations before and after application of the additional $^{1}/_{10}$ NB feeding are shown in Figure 37. Although all strains can grow on nutrient broth, *E. brevis* MT 2 exhibited the fastest optical density increase (see Chapter 3.3.1.1). The DOC increased to approximately 100 mg C l^{-1}. This was not caused by accumulation of 4-CS. Probably not all $^{1}/_{10}$ NB was degraded. A higher DOC background value, 80 mg C l^{-1}, was also observed in the pre-experiment when the consortium was grown on $^{1}/_{10}$ NB as sole carbon source in fed-batch culture (see Chapter 3.3.1.3, Figure 28). For this

experiment, no data for the occurrence of protoanemonin or *cis*-dienelactone was available due to an interference of unknown source in the HPLC measurement.

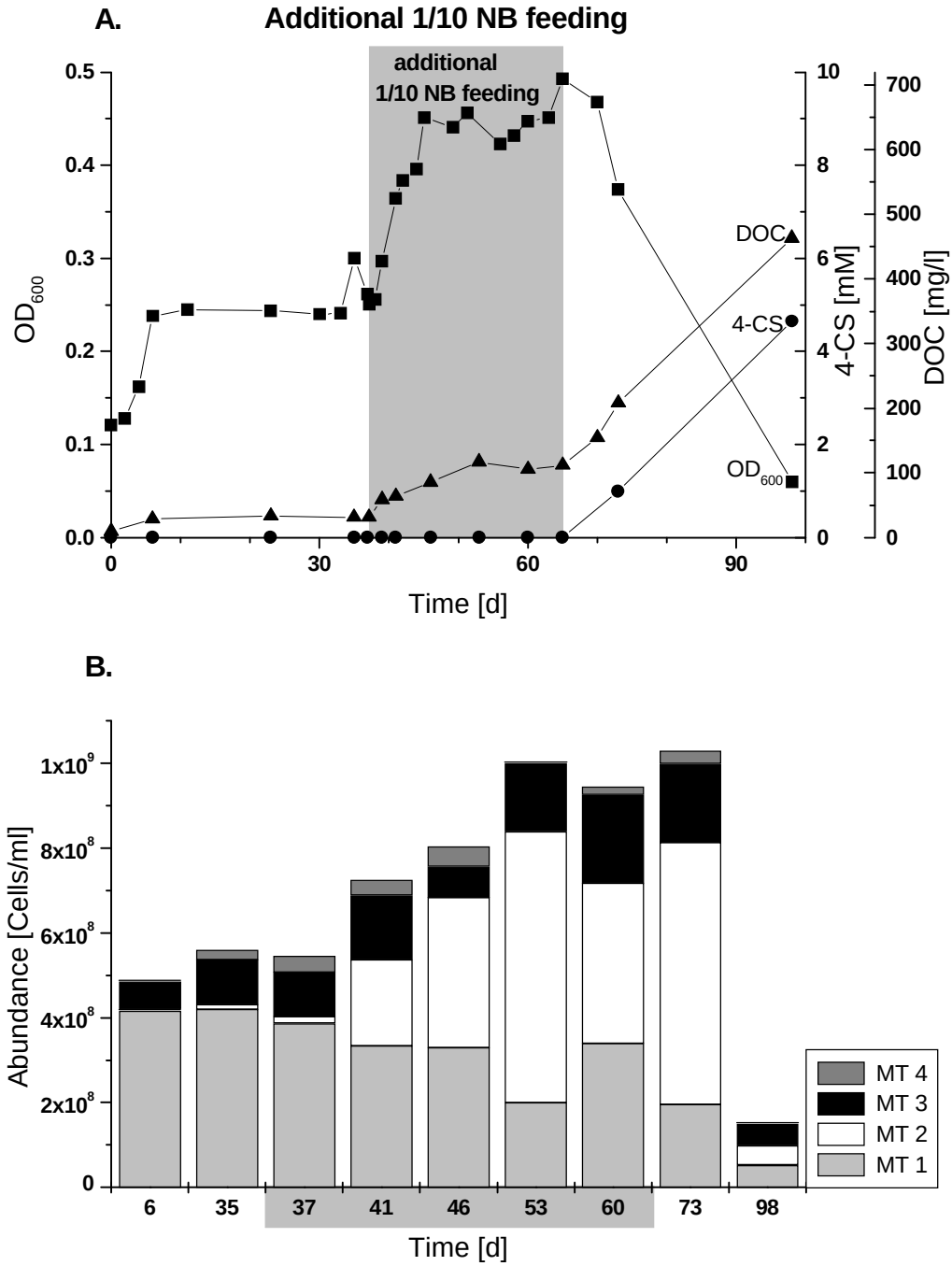

Figure 35: Consortium disturbed by additional feeding of $^{1}/_{10}$ NB A. Cell number (■), 4-CS concentration (●) and DOC (▲), B. Absolute abundance of consortium members

Frech also encountered problems with the HPLC measurement, when 4-CS was substituted by $^{1}/_{10}$ NB as feedstock (Frech, 1996). Between day 53 and 63, the DOC did not increase further and remained stable at a value of 100 (mg C) l^{-1}. In this time span was the abundance of *E. brevis* MT 2 approximately 50% of the consortium structure.

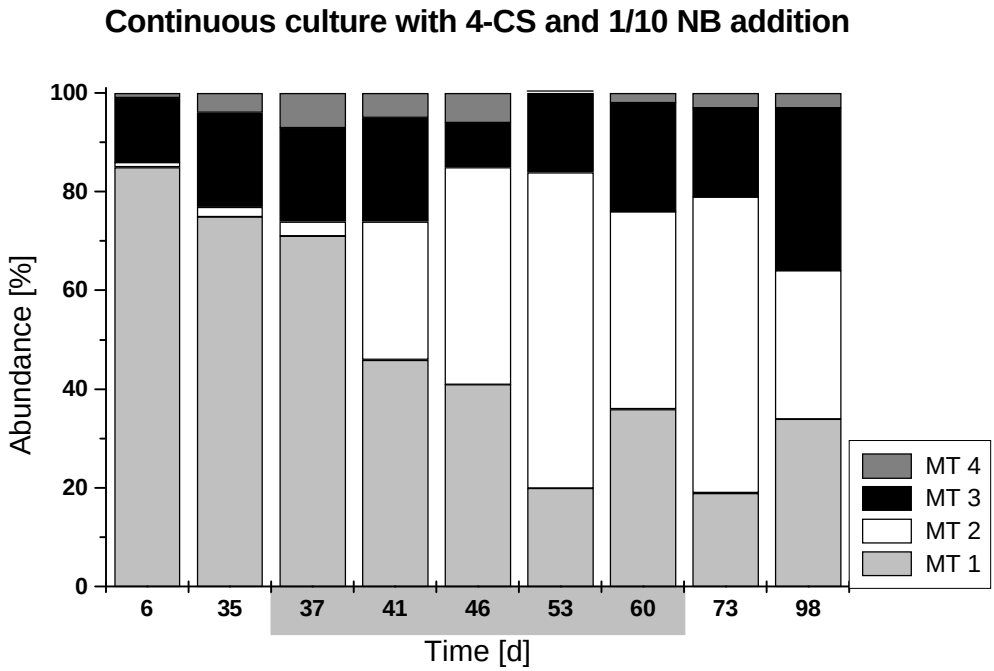

Figure 36: Consortium disturbed by additional feeding of $^{1}/_{10}$ NB; relative abundance of consortium members

The consortium structure changed to MT 1 = 32% MT 2 = 49%, MT 3 = 16% and MT 4 = 3% (Figure 36) (average of measurements taken on days 46, 53 and 60). Between day 53 and 63 parallel degradation of both carbon sources occurred. *E. brevis* MT 2 degraded the most of the nutrient broth, and the other three strains degraded 4-CS. When the additional feeding of $^{1}/_{10}$ NB was stopped, 4-CS was no longer degraded. This is a surprising observation. There seems to be no reason why the consortium should not be able to continue 4-CS degradation. If the system breakdown would result from the addition of $^{1}/_{10}$ NB, this influence should have been observed soon after the application of the disturbance and not when the disturbance was taken away.

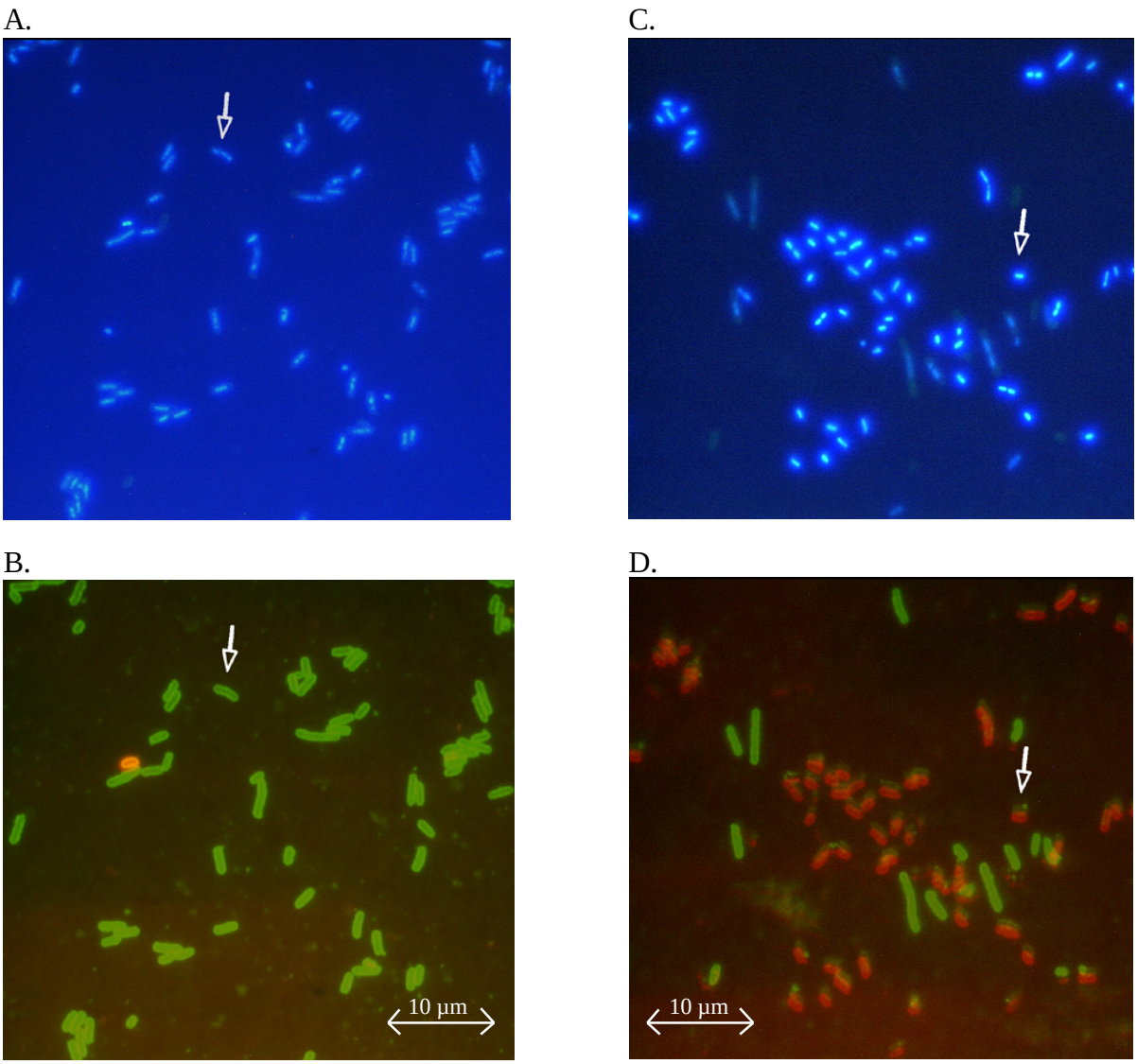

Figure 37: Consortium cells A, B previous to and C, D after additional $^{1}/_{10}$ NB feeding, stained with DAPI (A, C) or Cy 3 and DTAF (B, D)

3.3.3 Summary of mixed substrate utilisation experiments

In all mixed substrate continuous culture experiments, the results of the consortium structure under the influence of the disturbance and the functional effect of the disturbance matched very well with the results of the pre-experiments (see Table 10).

Table 10: Consortium structure and effect of second substrate addition of continuous culture compared to pre-experiment results

substrate	experiment	day	consortium structure				effect
			MT 1	MT 2	MT 3	MT 4	
4-CS + ethanol	pre-experiment [a]	4 [d]	24%	2%	17%	57%	washout
	continuous culture	16 [d]	26%	1%	12%	60%	washout
4-CS + iso-propanol	pre-experiment [a]	4 [d]	54%	1%	7%	38%	washout
	continuous culture	15 [d]	59%	1%	8%	32%	washout
4-CS + histidine	pre-experiment [b]	6 [e]	36%	1%	11%	52%	sim. degr.
	continuous culture	19	44%	2%	11%	44%	sim. degr.
$^1/_{10}$ NB	pre-experiment [a]	5 [e]	7% [f]	55% [f]	3% [f]	0% [f]	-
	(Frech, 1996)[c] continuous culture		10%	55%	15%	20%	-
4-CS + $^1/_{10}$ NB	continuous culture	[g]	32%	49%	16%	3%	sim. degr.

[a] fed-batch pre-experiment; [b] batch pre-experiment; [c] in this thesis the continuous culture experiment was performed with 4-CS and $^1/_{10}$ NB feeding, therefore the data of the pre-experiment is compared to the data of a continuous culture experiment with $^1/_{10}$ NB as sole carbon source performed by Frech (Frech, 1996); [d] before washout; [e] at the end of the experiment; [f] sum of percents is not 100% due to contamination; [g] average of days 46, 53 and 60;

3.4 Addition of optimised degraders

The 4-CS degrading consortium is a model community simulating environmental *in situ* situations where communities are responsible for degradation of pollutants. Invading organisms, which compete with one of the consortium members, could affect such functions dramatically. The aim of this chapter was to study whether single strains (having similar, or superior degradative abilities compared to single consortium members) can compete with the whole consortium or single consortium members.

Strain *P*. sp. MT 1 as only consortium member contains the enzyme salicylate 1-hydroxylase beside a catechol *ortho*-cleavage pathway (Pelz, 1999a). Thus *P*. sp. MT 1 is the only strain of the consortium capable to activate chlorosalicylate for ring-cleavage, but it contains a central metabolic route assumed to be not optimal for chlorocatechol degradation. Similarly, *P*. sp. MT 4 contains only an 3-oxoadipate pathway and this organism was assumed to support mineralisation of protoanemonin. Only strain *A. xylosoxidans* MT 3 has the chlorocatechol *ortho*-cleavage pathway genes and thus an enzyme equipment supposed to be optimal for chlorocatechol mineralisation. The combined activity of the three strains results in a better degradation rate for 4-CS as compared to *P*. sp. MT 1 (see Chapter 3.1.4 and 4.1). As competitors, three strains were selected: the genetically engineered strains, *Pseudomonas putida* G7::4/4 and *Pseudomonas* sp. B13 SN45P and an environmental isolate *Pseudomonas putida* A02.

The selection of the competitors was based on the kind of how the enzyme composition was generated (genetically engineered or by natural selection). Genetical engineering resulted in the successful broadening of the substrate range of microbial strains (as mentioned in chapter 1.2.1). These optimised stains can be introduced into a polluted site for the enhancement of degradation activities. The field performance of these GEMs (Schwieger & Tebbe, 2000) (and often also the performance in microcosms (Filonov et al., 1999; Nusslein et al., 1992; Watanabe et al., 1998)) has been inconsistent. Several explanations have been put forward to explain why GEMs should be unfit (Lenski, 1993). One explanation is that a genetically modified microorganism may be disadvantaged in the wild because of energetic costs associated with carriage and expression of additional genes (Brill, 1985). But the effects of genetic modifications on fitness are often highly dependent on environmental circumstances. Some studies support the supposition that expression of unnecessary metabolic function impairs fitness (Dykhuizen & Davies, 1980). On the other hand an introduced trait, which is beneficial for the microorganism in an specific environment (for example an bacterium that has been engineered to catabolize a pollutant released in environments where the pollutant is found in high concentrations) can enhance the fitness of the strain. In this thesis the competitiveness of two genetically modified strains under these two circumstances was studied.

3.4.1 Strain history

3.4.1.1 Constructed strain *P. putida* G7::4/4

Pseudomonas putida G7 is one of the best studied naphthalene degraders and genes encoding enzymes for naphthalene degradation are encoded on plasmid NAH7 (Dunn & Gunsalus, 1973; Yen & Gunsalus, 1982). The NAH genes are organised into two operons: encoded enzymes responsible for transformation of naphthalene into salicylate (upper pathway) and a second one encoding salicylate 1-hydroxylase and a catechol *meta*-cleavage pathway (lower pathway). *Pseudomonas putida* G7 is capable to cometabolize chloronaphthalenes and chlorosalicylate, but cannot grow on chlorosubstituted substrate derivatives due to the absence of a chlorocatechol pathway. A mini-transposon, containing the chlorocatechol *ortho*-cleavage genes of strain *Pseudomonas putida* P51, was introduced into the genome of *Pseudomonas putida* G7 by Jakobs, generating *P. putida* G7::4/4 (Jakobs, 1997). This strain is capable to mineralise 3-, 4- and 5-chlorosalicylate by a patchwork of NAH7 encoded salicylate 1-hydroxylase (Lehrbach et al., 1984) and chlorocatechol pathway enzymes.

In the chemostat environment with 4-CS as sole source of carbon and energy is the "additional genetic package" of *P. putida* G7::4/4 beneficial. The strain does not only contain the salicylate 1-hydroxylase (like *P.* sp. MT 1) but also the chlorocatechol pathway enzymes (like *A. xylosoxidans* MT 3). These combination of pathways is superior to the genetical composition of the single consortium members.

3.4.1.2 Constructed strain *P.* sp. B13 SN45P

The strain *Pseudomonas* sp. B13 SN45P, is a derivative of *P.* sp. B13 (Dorn et al., 1974), and, like the parent, contains chlorocatechol genes. *P.* sp. B13 SN45P differs from the parent by its capability to grow on mixtures of 4-chlorobenzoate and 4-methylbenzoate (Müller et al., 1996), due to the introduction of genes derived from the archetype TOL plasmid pWWO encoding toluate dioxygenase and dihydrodiol dehydrogenase and of a gene encoding 4-methylmuconolactone methylisomerase from *Ralstonia eutrophus* JMP 134 (Müller et al., 1996; Rojo et al., 1987). However, both *P.* sp. B13 as well as *P.* sp. B13 SN45P are not able to mineralise chlorosalicylates. Thus, *P.* sp. B13 SN45P would compete with the consortium member *A. xylosoxidans* MT 3.

In this strain, the trait which was additionally introduced into *P.* sp. B13 SN45P is not necessary for the ability to mineralise 4-chlorocatechol; it is an example of "additional genetic package", which is superfluous for the microbial strain.

3.4.1.3 Environmental isolate *P. putida* A02

The strain *P. putida* A02 was isolated from the Elbe River, Germany and donated by Dr. Pieper. 16S rDNA analysis revealed that the strain was closely affiliated to *Pseudomonas*

putida. The strain was able to growth on 4-CS plates, which indicated the ability to activate 4-CS. The specific enzymatic activities of the ring-cleavage enzymes were tested to reveal whether the strain contains the chlorocatechol *ortho*-cleavage enzymes (as described 2.7.2). Strain *Pseudomonas* sp. RW 10, which, like *P.* sp. MT 1, contains only 3-oxoadipate pathway enzymes, was used as control.

Table 11: Specific activities of catabolic enzymes in cell extracts of *Pseudomonas* sp. RW 10 and *Pseudomonas putida* A02, grown on 4-CS

Enzyme activity	**Assay substrate**	**Specific activity** (U g^{-1} protein) of strain	
		RW 10	**A02**
Catechol 1,2-Dioxygenase	Catechol	472 (100%)*	827 (100%)*
	3-Chlorocatechol	13 (3%)*	974 (120%)*
	4-Chlorocatechol	178 (37%)*	717 (87%)*

* Relative enzyme activities are expressed as percentages of that for unchlorinated catechols (=100%);

Chlorocatechol 1,2-dioxygenase and catechol 1,2-dioxygenase differ only in substrate specificity. The enzymes can be distinguished by the fact that pyrocatechase II (or chlorocatechol 1,2-dioxygenase) showed high relative activities with 3- and 4-chlorocatechol compared with catechol (Dorn & Knackmuss, 1978a; Dorn & Knackmuss, 1978b). Dorn and Knackmuss found that the activity of the catechol 1,2-dioxygenase was 0.7% with 3-CC and 11.3% with 4-CC as substrate, whereas the activity of the chlorocatechol 1,2-dioxygenase was 105% with 3-CC and 96% with 4-CC as substrate, as percentages of enzyme activities for unchlorinated catechols (Dorn & Knackmuss, 1978b). The low enzyme activities against 3-chlorocatechol (3-CC) in *P.* sp. RW 10 (see Table 11), show that the strain contains just the 3-oxoadipate pathway, whereas the high enzyme activity against 3-CC in *P. putida* A02 is indicative for the chlorocatechol pathway.

P. putida A02 contained a similar enzymatic composition as did *P. putida* G7::4/4. *P. putida* A02 is an environmental isolate and not a GEM. *P. putida* A02 was introduced as competitor because GEMs are thought to be less fit in the competition with natural microflora, as they carry a heavier genetic package.

Due to its better enzymatic composition it was expected that *P. putida* A02 would outcompete the whole consortium or substitute one of the consortium members.

3.4.2 Batch cultures of pure cultures of two competitors

Batch cultures with the strains *P. putida* A02 and *P. putida* G7::4/4 were used to test the degradative abilities of these strains. *P. putida* A02 was grown in minimal medium with

2.5 mM 4-CS, and *P. putida* G7::4/4 was grown in minimal medium containing 5 mM 4-CS as the sole source of carbon and energy as described in 2.5.3.2. For the calculation of the biomass, the equation (mg dw) $l^{-1} = 0.92 + 656.9 \times OD_{578}$ (from 2.6.1) was applied.

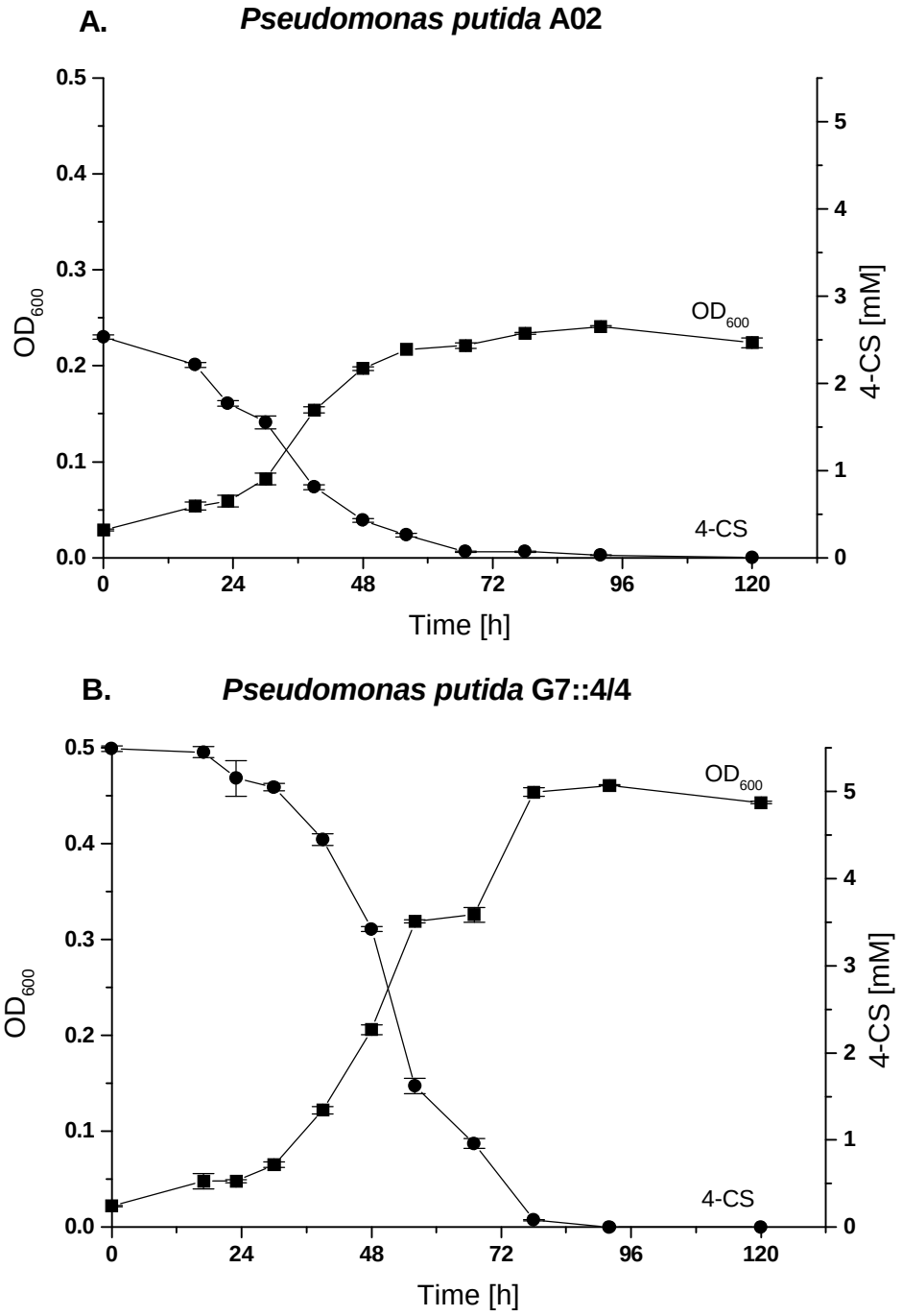

Figure 38: Optical density (■) and 4-chlorosalicylate concentration (●) of growth of A. *P. putida* A02 and B. *P. putida* G7::4/4 on 4-CS in batch culture. The given values are the mean of 2 independent experiments.

Batch culture growth of *P. putida* A02 on 2.5 mM 4-CS and *P. putida* G7::4/4 on 5 mM 4-CS is shown in Figure 38. Calculation of the yield, growth rate and degradation rate was performed as described for *P.* sp. MT 1 in 2.6.4. The growth rate of *P. putida* A02 with

2.5 mM 4-CS was 0.038 h^{-1} and the degradation rate 0.828 mM l^{-1} (g dw)$^{-1}$ and the yield of 0.32, the growth rate of *P. putida* G7::4/4 with 5 mM 4-CS was 0.058 h^{-1} and the degradation rate 1.28 mM l^{-1} (g dw)$^{-1}$, the yield was 0.31. In comparison, the main degrader of the consortium, *P.* sp. MT 1, had a growth rate of 0.019 h^{-1} and a degradation rate of 0.234 mM l^{-1} (g dw)$^{-1}$ and a yield of 0.37, when grown in batch culture on 1 mM 4-CS. Therefore, the competing strains have higher growth and degradation rates. Additionally have the competing strains a greater potential to tolerate the 4-CS concentration in batch culture. Inhibition was observed for *P.* sp. MT 1 and *P. putida* A02 at 4-CS concentrations higher than 2 mM and 3.5 mM, respectively, *P. putida* G7::4/4 did grow undisturbed at a concentration of 5 mM 4-CS.

3.4.3 Competitor addition experiments in continuous culture

Cells of *P. putida* A02 and *P. putida* G7::4/4, respectively, were pre-grown in batch culture, washed and then added to the consortium, which was growing in continuous culture under the standard conditions, as described in 2.5.3.4.

The undisturbed control is shown in Chapter 3.3.2, Figure 33. 4-CS was completely degraded during the whole experiment, and no accumulation of *cis*-dienelactone was observed. Only minor insignificant shifts in the OD_{600} and the consortium structure were detected. On day 32-35 a higher optical density was observed, which was due to additional medium influx created by the application of disturbances in the other chemostats.

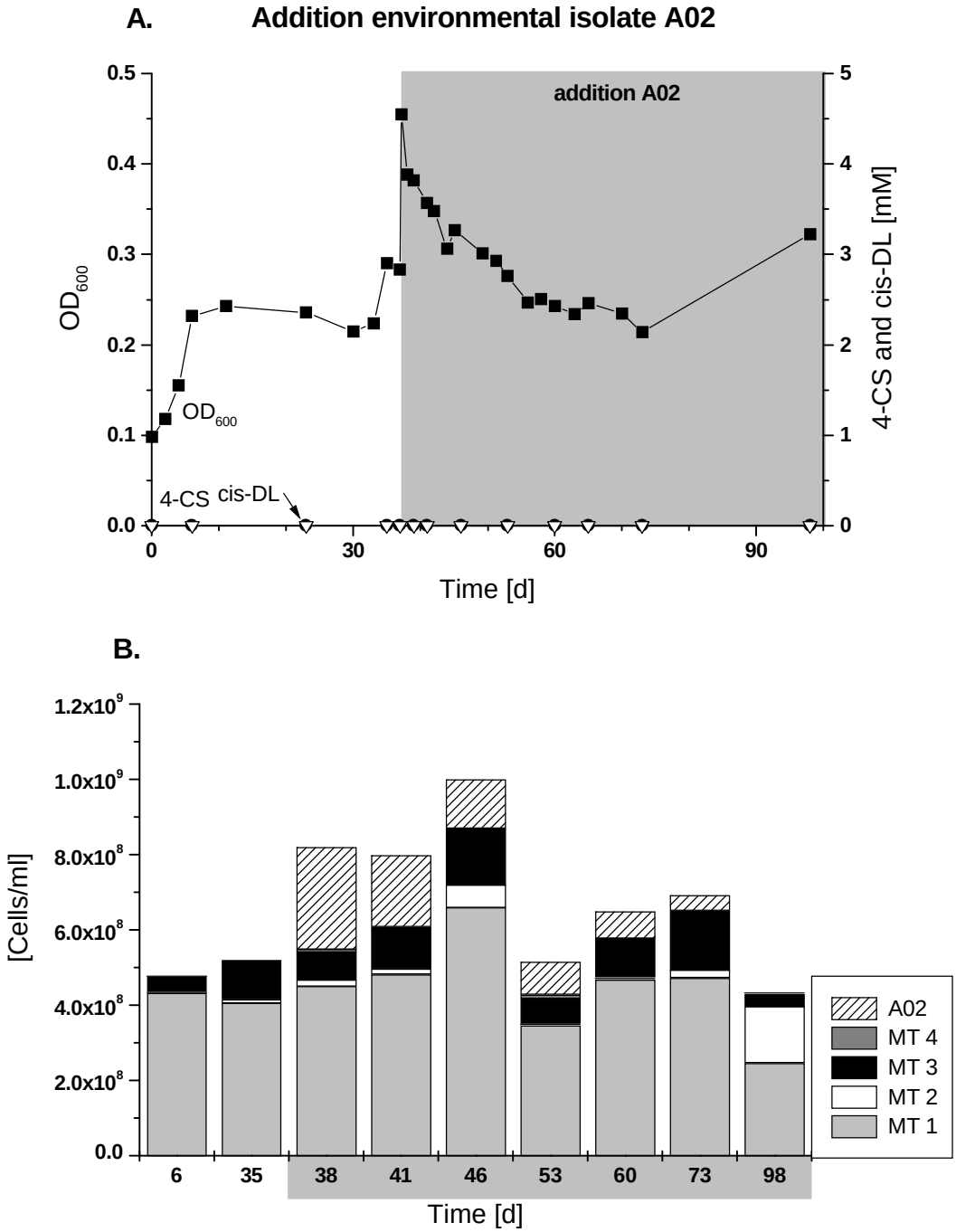

Figure 39: Continuous culture of consortium, disturbed by addition of cells of strain *P. putida* A02, A. OD_{600} (■), 4-CS (●) and *cis*-dienelactone (▽) concentration , B. absolute abundance of strains

The effect of the addition of environmental isolate *P. putida* A02 upon the cell concentration and consortium structure is shown in Figure 39. The consortium was grown in continuous culture without disturbance for 30 days and three volume exchanges ensured the generation of a steady state. Due to the addition of the competitor cells, the optical density doubled. As the feed still contained 5 mM 4-CS, which can only sustain cell densities of an optical density of approximately 0.25, the OD decreased thereafter, and after 30 days optical density similar to

that before the addition of *P. putida* A02 was reached. Monitoring of the absolute abundance of community members showed that addition of the competitor *P. putida* A02 on day 38 resulted in a doubling in cell number, but that *P. putida* A02 was then washed out (Figure 39B). At day 98 the concentration of the *P. putida* A02 cells was 6.5×10^6 cells ml^{-1} (1% in abundance of the consortium cell number; 2% in abundance of the *P. putida* A02 cell number on day 38). The average consortium composition could not be assessed, as the consortium composition was always changing due to the decrease in *P. putida* A02 cell number. For example, the consortium composition on day 73 was MT 1 = 69%, MT 2 = 3%, MT 3 = 23%, MT 4 = 0%, A02 = 6%. Compared to the undisturbed consortium composition, the abundance of *P.* sp. MT 1 and *P.* sp. MT 4 was lower. Possible explanations are that competition occurred between *P. putida* A02 and *P.* sp. MT 1 and that less protoanemonin was excreted, which resulted in lower concentrations of *P.* sp. MT 4.

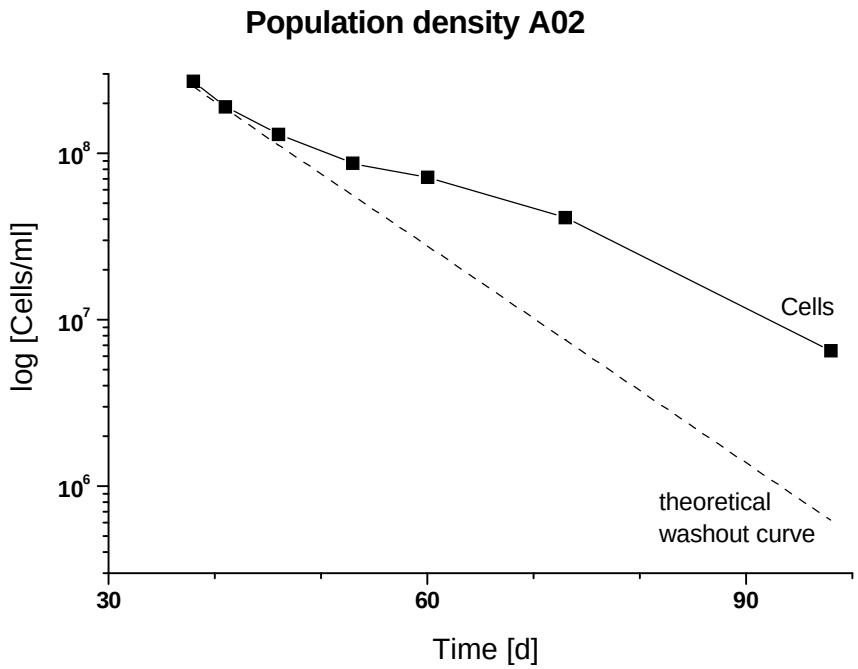

Figure 40: Dynamic of *P. putida* A02 population introduced to consortium growing in continuous culture at D = 0.1 and 5 mM 4-CS in feedstock, population density of A02 (■), dashed line, theoretical washout curve;

The changes in population density of strain *P. putida* A02 are shown in Figure 40 and compared to the theoretical washout curve, which would occur without growth of *P. putida* A02. The resulting curve was less steep than the washout curve, which indicates cell growth, but the competitor concentration was continuously decreasing up to the end of the experiment. The growth rate of *P. putida* A02, which can be deduced from the density curve revealed a growth rate of $\mu = 0.022\ h^{-1}$, this is lower than the dilution rate of $0.026\ h^{-1}$. The growth rate of *P. putida* A02 was lower, therefore washout occurred. Additionally was the growth rate lower as the growth rate in batch culture with 2.5 mM 4-CS, which was $0.039\ h^{-1}$. Probably had the strain not enough access to the carbon source.

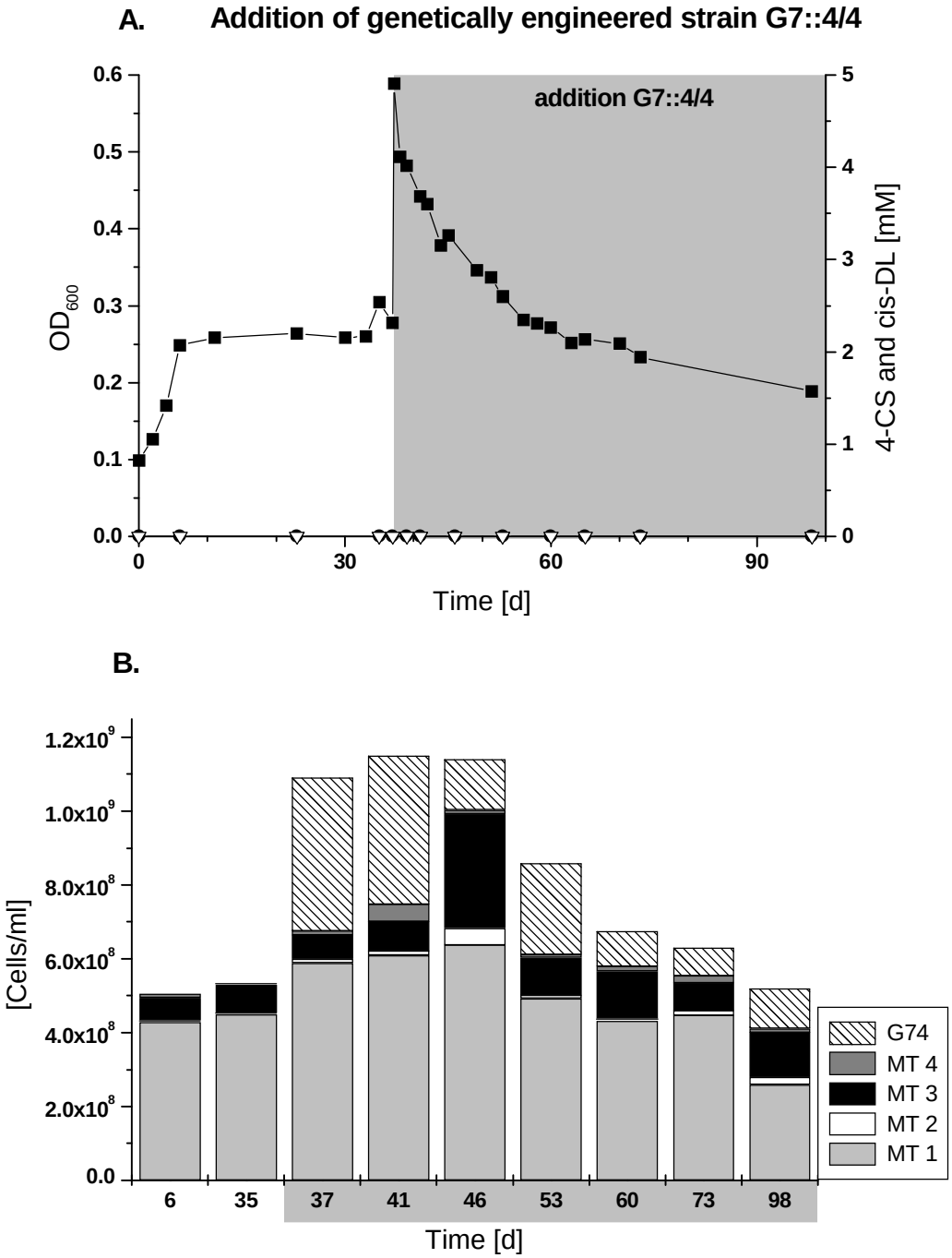

Figure 41: Continuous culture of consortium, disturbed by addition of cells of strain *P. putida* G7::4/4, A. OD_{600} (■), 4-CS (●) and *cis*-dienelactone (▽) concentration, B. absolute abundance of strains

The effect of the addition of competitor *P. putida* G7::4/4 upon the cell concentration and consortium structure is shown in Figure 41. The addition of the competitor on day 37 resulted, as expected, in doubling of the cell number. As the feed still contained 5 mM 4-CS, the OD decreased thereafter. During the further time course of the experiment cell number declined to 5×10^8 cells ml^{-1} (see Figure 42), as observed in the undisturbed community growing on 5 mM 4-CS. However, in contrast to *P. putida* A02, *P. putida* G7::4/4 established itself as a

stable community member. As shown in Figure 42, after an initial washout, *P. putida* G7::4/4 cell numbers remained constant at 10^8 cells ml^{-1}, comprising 14% of the total community. The abundance of MT 1 in the here established consortium was significantly lower than in the undisturbed consortium. The abundance of *P.* sp. MT 1 (63%) and of *P. putida* G7::4/4 (14%) after addition of the competitor sum up to the abundance of *P.* sp. MT 1 before (76%), indicating *P. putida* G7::4/4 to successfully compete with *P.* sp. MT 1. The abundance of *P.* sp. MT 4 was also significantly decreased. But the low concentration of *P.* sp. MT 4 was based on a generally lower concentration of *P.* sp. MT 4 during this set of experiments, which can be seen in the undisturbed experiment (see Figure 33 and Figure 34) and the consortium composition before addition of *P. putida* G7::4/4 (Figure 41).

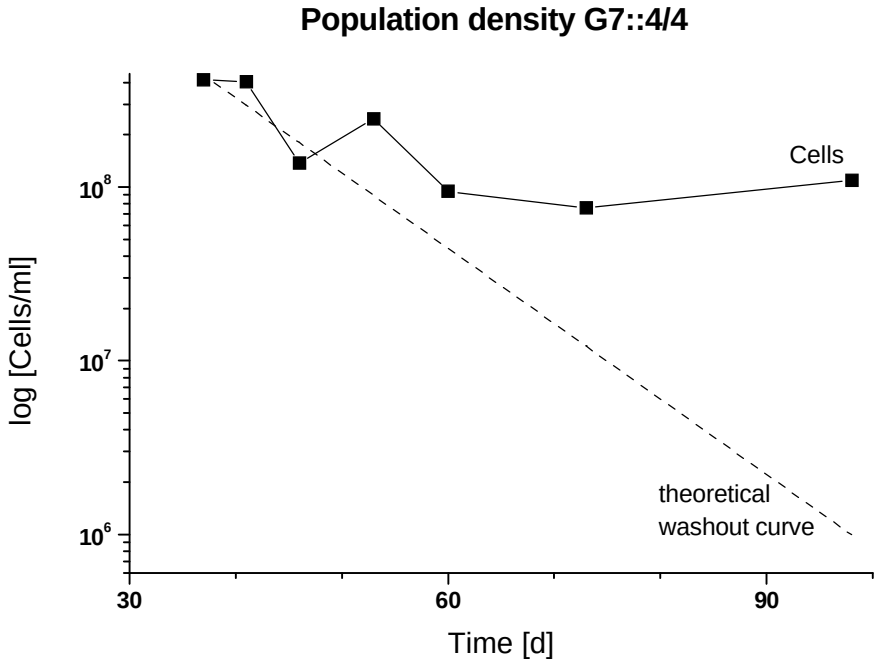

Figure 42: Dynamic of *P. putida* G7::4/4 population introduced to consortium growing in continuous culture at D = 0.1 and 5 mM 4-CS in feedstock; population density of G7::4/4 (■); dashed line, theoretical washout curve;

3.4.4 Influence of two factors (carbon source and optimised degraders) upon consortium

When, as shown above, in Chapter 3.4.3, the competitors were added to the consortium growing on 5 mM 4-CS without an increase in carbon supply, the cell number decreased thereafter to pre-disturbance conditions. In the here displayed experiments, additional carbon source was supplemented to enable parallel growth of the competitor and consortium. In the previous experiment the influence of one disturbance (addition competitor) was tested. Now the influence of two disturbances (addition carbon source and addition competitor) were studied.

3.4.4.1 Consortium growth on 10 mM 4-CS and on 10 mM 4-CS with addition of *P. putida* G7::4/4

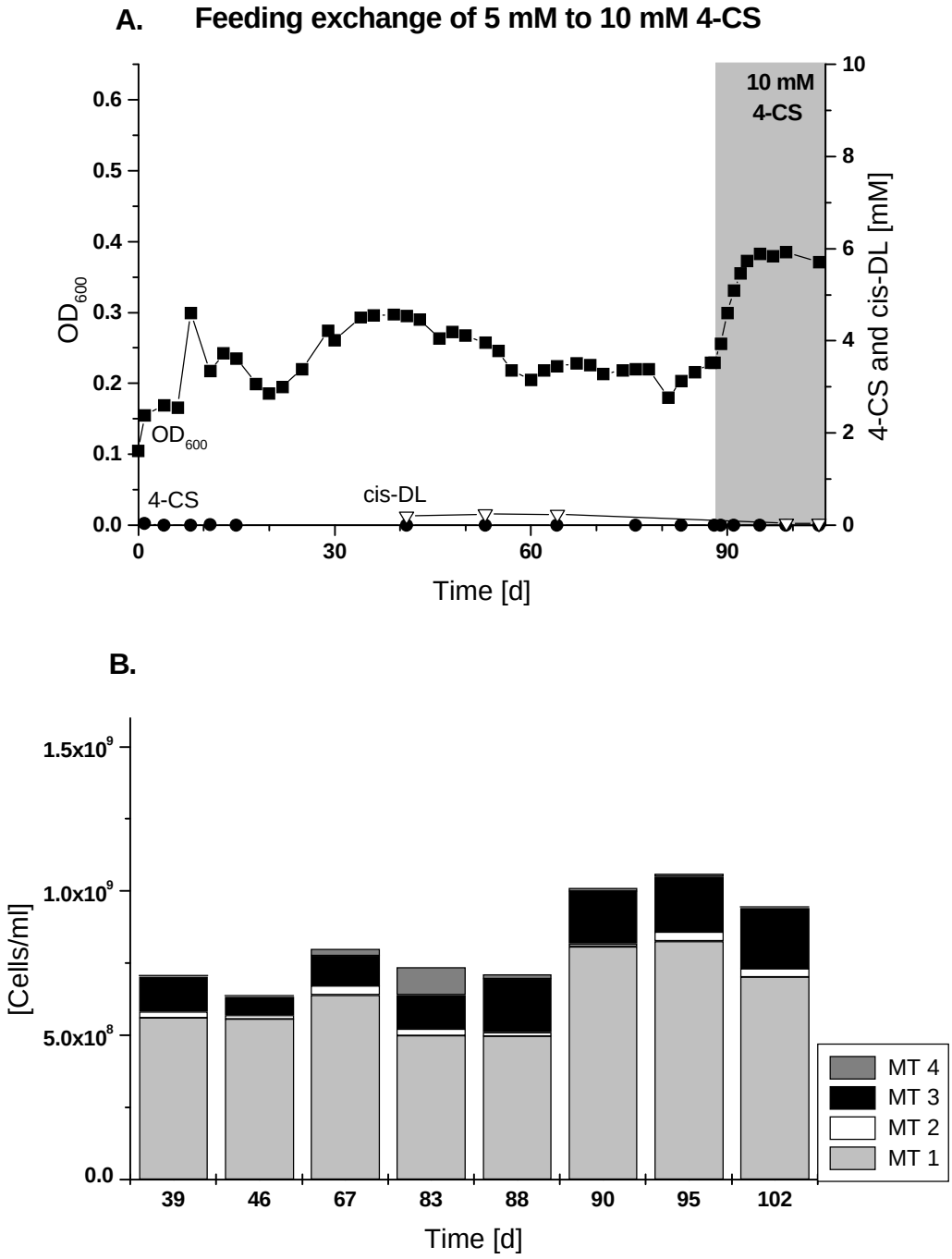

Figure 43: Consortium disturbed by doubling amount of 4-chlorosalicylate in feedstock from 5 mM to 10 mM A. Optical density (■), 4-CS (●) and *cis*-dienelactone (▽) concentration; B. Absolute abundance of consortium members

First the reaction of the consortium to the increase of the carbon source concentration to 10 mM 4-CS was studied, because this concentration might be toxic for the consortium or influence consortium composition or function. The undisturbed control (consortium growth on 5 mM 4-CS in continuous culture) of this set of experiments is shown in the Appendix, Chapter 6.3, Figure 58. The long pre-disturbance run phase had no negative effect upon the

consortium, as the cell concentration was 5×10^8 cells ml^{-1}, as expected for the undisturbed steady state (see Chapter 3.2.1), and all 4-CS was degraded. But a difference in the consortium composition was observed, the abundance of *P.* sp. MT 4 was lower than usual.

The effect of the increase of 4-CS concentration in the feedstock from 5 mM to 10 mM is shown in Figure 43. The doubling of the carbon source concentration resulted as expected in an increase of the cell number. The average relative abundance from day 88 until the end of the experiment was MT 1 = 76%, MT 2 = 2%, MT 3 = 21% and MT 4 = 1% (Figure 43B). This corresponds to the expected abundance of the consortium members under undisturbed conditions shown in Chapter 3.2.2. This experiment shows the ability of the consortium to degrade 10 mM 4-CS in continuous culture.

Second the consortium was not only stressed by the increase of the carbon source from 5 mM to 10 mM 4-CS, but also by addition of a new strain, *P. putida* G7::4/4, which was pregrown in batch culture on 5 mM 4-CS.

The effect of the increase of 4-CS concentration in the feedstock from 5 mM to 10 mM and of the addition of cells of strain *P. putida* G7::4/4 is displayed in Figure 44. When the concentration of 4-CS was doubled and strain *P. putida* G7::4/4 was added, doubling of the cell number resulted (see Figure 44A). A new consortium composition, with *P. putida* G7::4/4 as consortium member generated, this can be seen in Figure 44B and Figure 45. The consortium composition was MT 1 = 59%, MT 2 = 3%, MT 3 = 14%, MT 4 = 1%, G7::4/4 = 23%. At the end of the experiment, on day 102, a very low number of *P. putida* G7::4/4 cells was observed (see Figure 45). This value can be compared to the theoretical washout curve. A cell number this low could not be reached, even when the cells were not growing at all. Therefore this value is an outlier. The result of the experiment was that *P. putida* G7::4/4 was able to survive in the consortium and became a new consortium member which neither outcompeted other consortium members nor dominated the consortium. The average concentration of *P. putida* G7::4/4 cells in this continuous culture was 3.2×10^8 cells ml^{-1}. This is three times the amount of *P. putida* G7::4/4 concentration which was reached by addition of the strain to the consortium growing on 5 mM 4-CS (Figure 41).

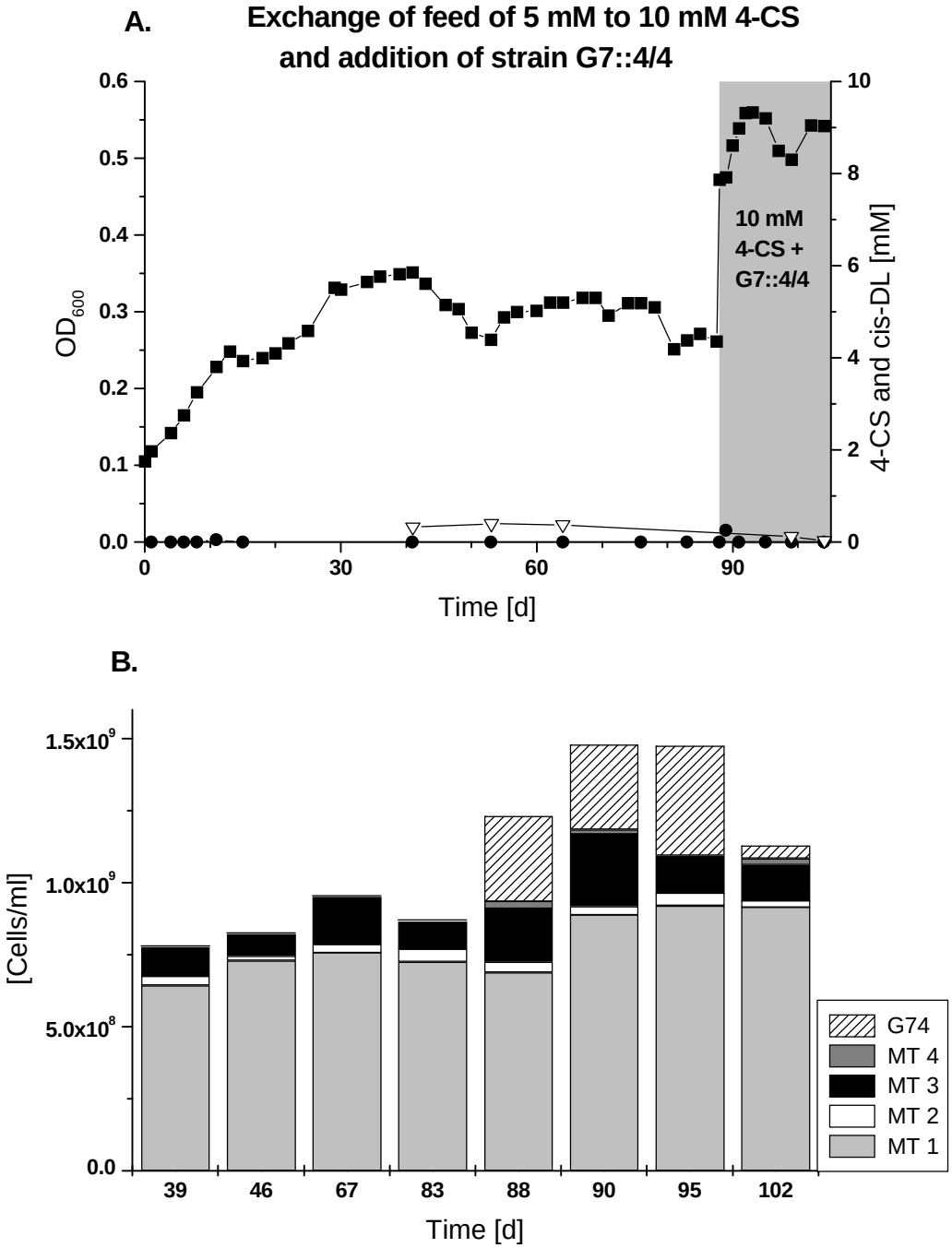

Figure 44: Consortium disturbed by doubling amount of 4-chlorosalicylate in feedstock from 5 mM to 10 mM and addition of cells of strain *P. putida* G7::4/4; A. Optical density (■), 4-CS (●) and *cis*-dienelactone (▽) concentration; B. Abundance of consortium members

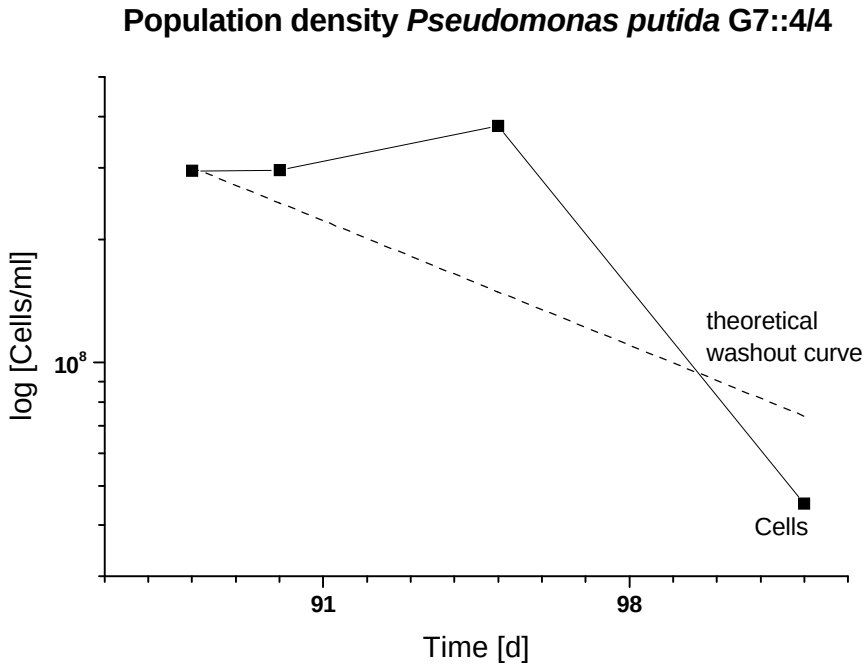

Figure 45: Population development of *P. putida* G7::4/4 population added to continuous culture of 4-CS degrading consortium with exchange of feedstock of 5 mM 4-CS to 10 mM 4-CS as carbon source; population density of G7::4/4 (■); dashed line, theoretical washout curve

3.4.4.2 Consortium growth on mixture of 5 mM 4-CS and 5 mM 4-chlorocatechol and on this mixture with addition of *P.* sp. B13 SN45

5 mM 4-chlorocatechol (4-CC), an intermediate of the 4-CS degradation pathway, was fed in addition to 5 mM 4-CS. This substrate combination might be toxic for the consortium or at least be a stress factor. Therefore the influence of the carbon source change was studied first. The addition of 4-CC should lead to the degradation of 4-CC by *A. xylosoxidans* MT 3, due to results from Pelz (1999a). Pelz stated that *P.* sp. MT 1, *A. xylosoxidans* MT 3 and *P.* sp. MT 4 can use 4-CC as a carbon source, but C^{13} labelled 4-CC was, of all consortium members, incorporated fastest by *A. xylosoxidans* MT 3.

The following experiment can be compared to the undisturbed experiment shown in Chapter 3.2.1, Figure 14.

The continuous culture was grown under undisturbed conditions until steady state. After the addition of 5 mM 4-CC the biomass increased (see Figure 46). This was due to an increase in the abundance of strain *A. xylosoxidans* MT 3. This is in accordance with observation by Pelz et al. of *A. xylosoxidans* MT 3 to rapidly incorporate 4-CC (Pelz, 1999a). *P.* sp. MT 1 did not increase in cell number, as can be seen in the plot of absolute abundance in Figure 47. The consortium structure changed to MT 1 = 68%, MT 2 = 1%, MT 3 = 30%, and MT 4 = 2%, as can be seen in Figure 46B.

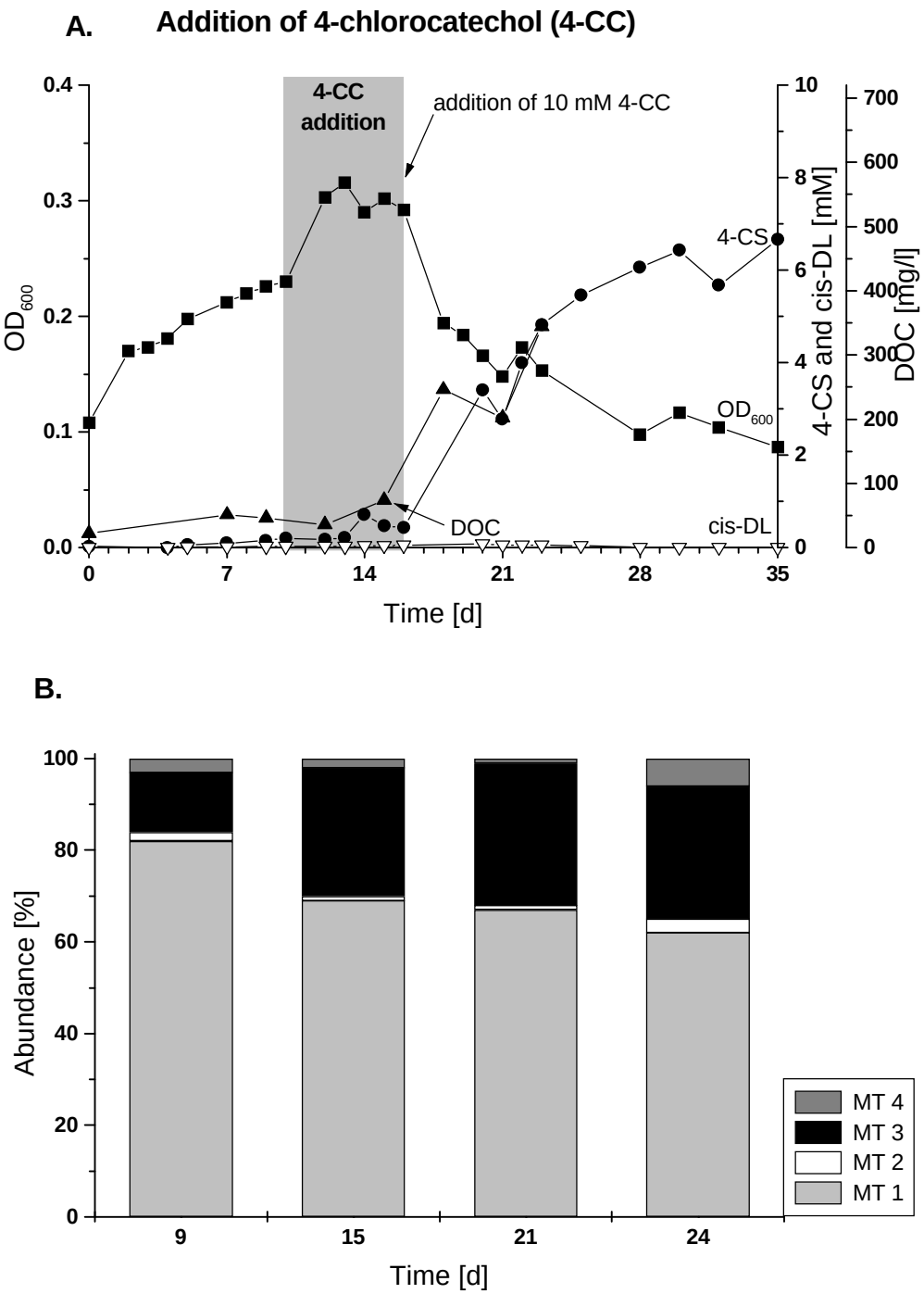

Figure 46: Consortium growing on 5 mM 4-CS in continuous culture, disturbed by 5 mM 4-CC addition A. Optical density (■), 4-CS (●) and *cis*-DL (▼) concentration and DOC (▲); B. Abundance of consortium members

During the first days after addition of 4-CC (day 10 and day 13) no accumulation of substrate nor intermediates was observed and the DOC remained at low levels similar to those observed under undisturbed conditions. Between day 13 and day 14, low amounts (0.8 mM) of 4-CS accumulated concomitant with a slight increase in DOC. This accumulation does not indicate a beginning system breakdown, as the concentration of accumulated 4-CS was no longer increasing, but was slowly decreasing between day 14 and 16. Until day 16 the chemostat had

its usual colour. This demonstrates that the consortium was able to degrade both carbon sources in parallel.

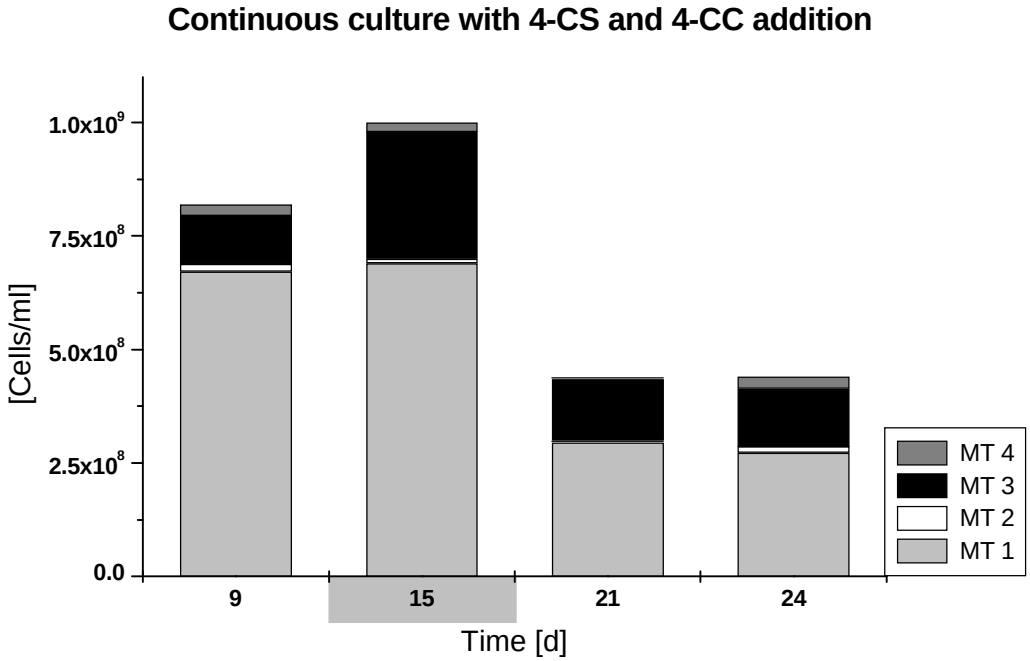

Figure 47: Absolute abundance of consortium growing on 5 mM 4-CS, disturbed by 4-chlorocatechol (4-CC) addition

At day 16 the 4-CC concentration in the feedstock was increased to 10 mM for one day and then reduced again to 5 mM for another two days, until day 18. The high amount of 4-CC could obviously not be metabolised anymore, breakdown of the culture was observed. Therefore the original carbon source, 4-CS, was not degraded and accumulated in the reaction vessel until the feedstock concentration was achieved. Between day 16 and 18, the DOC was higher than the concentration of 4-CS, due to the amount of 4-CC in the vessel. After day 18, the DOC measurement had the same value as 4-CS, which was the only carbon source still offered. The addition of 10 mM 4-CC resulted in a brown colouration of the reaction liquid. This was due to the build-up and condensation of 4-CC in the vessel. Difficulties in monitoring accumulated 4-CC were previously observed. Babu et al. observed catechol accumulation until 18 h, after which observation was no longer possible due to auto-oxidation and condensation (Babu et al., 1995). It is to be expected that the auto-oxidation and condensation of catechol would need the same time in the 4-CC addition experiment. As sampling was performed once a day it was not possible to measure the accumulated catechol by HPLC, but a DOC increase due to accumulating 4-CC should be measurable. As after day 16 no further DOC increase was observed, the main part of the 4-CC was still metabolised, certainly by *A. xylosoxidans* MT 3, because the abundance of this strain remained unchanged at day 21 and 24 (see Figure 47). The observed breakdown of the system was definitely due to the addition of too much 4-CC for one day. This must have influenced *P*. sp. MT 1, as the concentration of P. sp. MT 1 cells reduced dramatically after day 15 (see Figure 47) and 4-CS degradation stopped and a washout occurred.

In summary: The additional feeding of 4-CC led to an increase of the consortium member *A. xylosoxidans* MT 3. The absolute abundance of *P.* sp. MT 1 under the influence of the additional feeding of 4-CC (6.7×10^8 cells ml^{-1}) is higher than the abundance in undisturbed experiments (4.9×10^8 cells ml^{-1}), indicating that *P.* sp. MT 1 also metabolised 4-CC. The consortium was able to degrade 5 mM 4-CC and 4-CS in parallel. In addition it was possible to show how a system breakdown due to high amounts of 4-CC would occur.

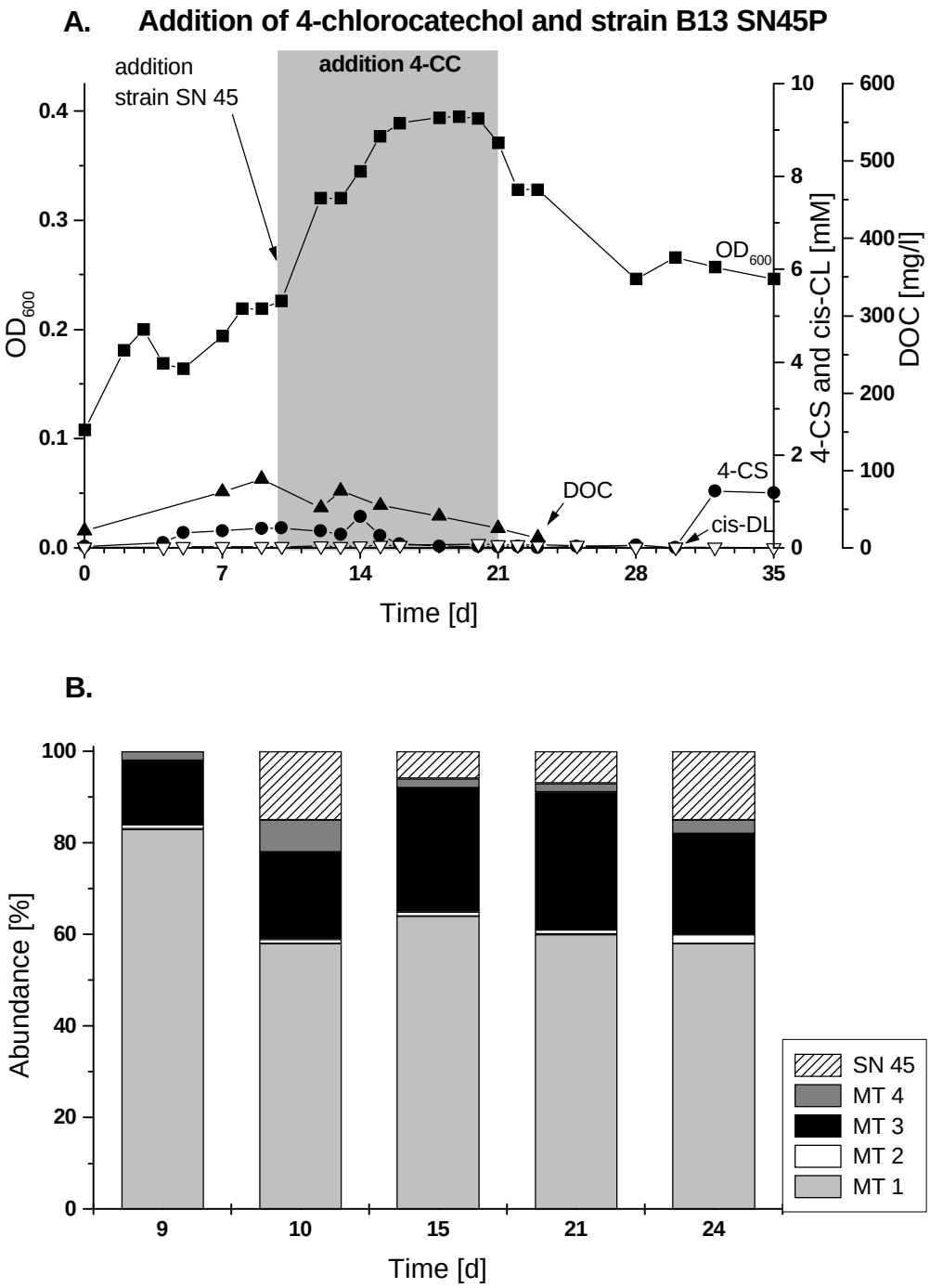

Figure 48. Consortium growing on 5 mM 4-CS in continuous culture, disturbed by addition of 5 mM 4-CC and cells of strain *P.* sp. B13 SN45P A. Optical density (■), 4-CS (●), *cis*-DL (▽) concentration and DOC (▲); B. Abundance of consortium members

In a parallel experiment, a stable continuous culture was supplemented with 5 mM 4-CS and 5 mM 4-CC as carbon source and strain *P.* sp. B13 SN45P (Figure 48). Strain *P.* sp. B13 SN45P was pregrown on nutrient broth in batch culture, because batch culture on 4-CC as carbon source is difficult because the substrate decays with time. The 4-CS concentration in the reaction vessel was 0.5 mM 4-CS, when the feeding of 4-CC started and the strain was added, but under the influence of the disturbance, the accumulation of 4-CS retreated and between day 23 and 30 no 4-CS was found in the vessel anymore, as all was consumed.
The addition of the second carbon source and *P.* sp. B13 SN45P cells resulted in an initial phase with increased optical density, due to the supplemented cells and to growth of *P.* sp. MT 1 between day 10 to 12 and growth of *A. xylosoxidans* MT 3 between day 13 to 15 (see Figure 48B and Figure 49 and Figure 50). Both carbon sources were degraded simultaneously. Between day 15 and day 21, a new steady state had been established with a consortium composition of MT 1 = 60%, MT 2 = 1%, MT 3 = 25%, MT 4 = 4%, B13 SN45P = 10%.

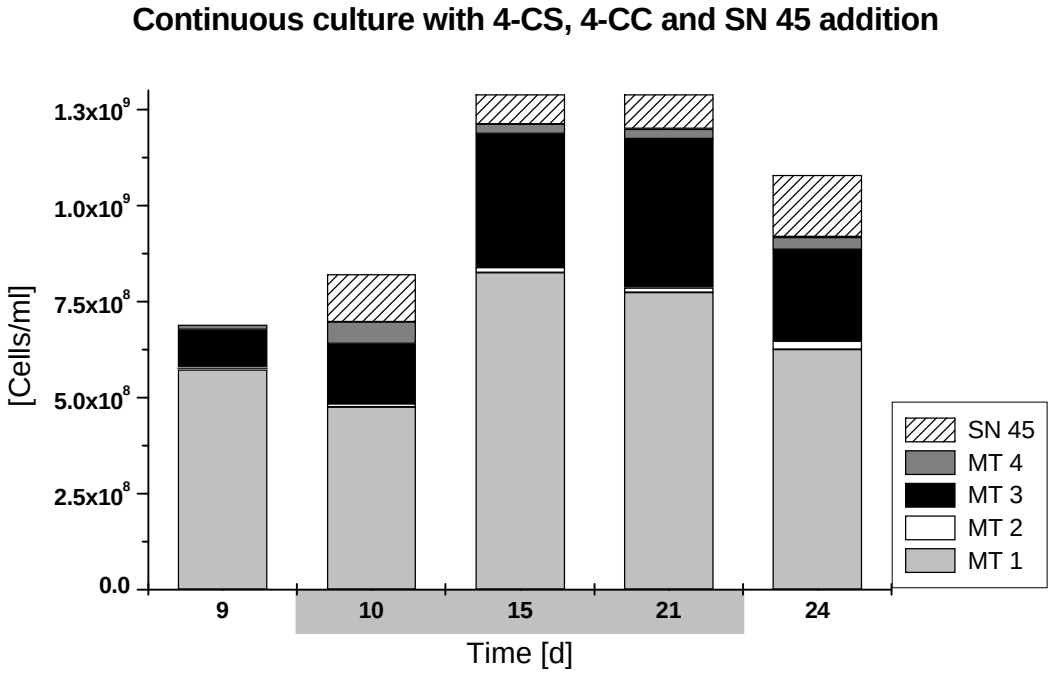

Figure 49: Absolute abundance of consortium growing on 5 mM 4-CS, disturbed by addition of 4-chlorocatechol (4-CC) and cells of the strain *P.* sp. B13 SN45P

The consortium structure of the previous experiment, in which only 5 mM 4-CC was added and no competitor, also resulted in higher relative abundance of the consortium member *A. xylosoxidans* MT 3 and in higher absolute abundance of *P.* sp. MT 1. The absolute abundance is shown in Figure 49. The optical density remained stable and all 4-CS was degraded. When 4-CC was no longer fed, the cell number diminished, as less carbon was available for growth. The abundance of *A. xylosoxidans* MT 3 and of *P.* sp. MT 1 reduced, but strain *P.* sp. B13 SN45P was still in abundance.

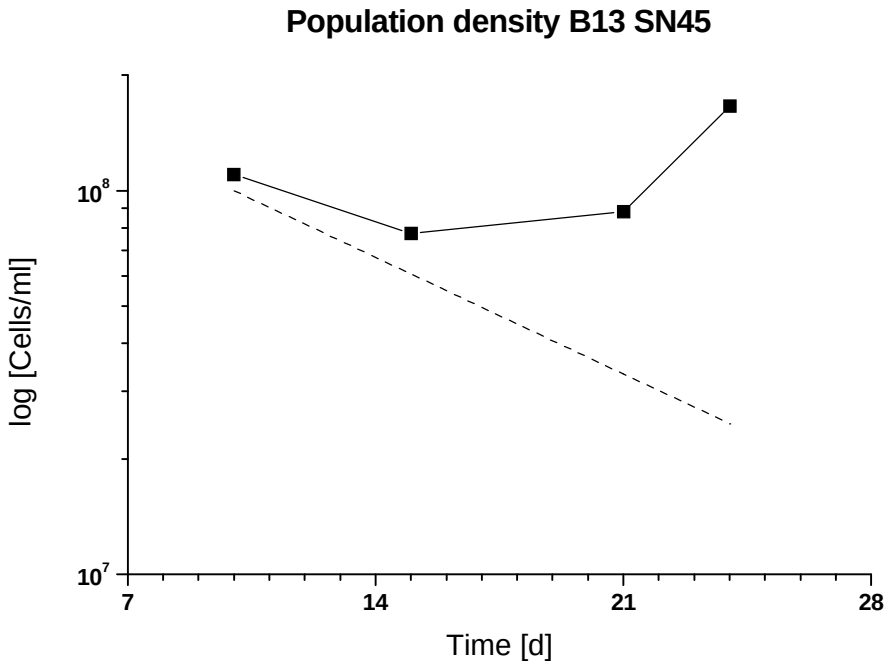

Figure 50: Population development of *P.* sp. B13 SN45P population added to continuous culture of 4-CS degrading consortium with 5 mM 4-CS and 5 mM 4-CC as carbon source; population density of B13 SN45P (■); dashed line, theoretical washout curve

In the *P.* sp. B13 SN45P population density plot (Figure 50), it is apparent that *P.* sp. B13 SN45P population was increasing in cell number even after stopping the feeding of the second carbon source, 4-CC. The strain *P.* sp. B13 SN45P should not be able to degrade 4-CS, as it does not contain the enzyme salicylate 1-hydroxylase. It is likely that both strains, *A. xylosoxidans* MT 3 and *P.* sp. B13 SN45P to live from the 4-CC spilling out from the 4-CS degradation of *P.* sp. MT 1. The 4-CS concentration increased to 1 mM between day 32 and day 35. The reasons for this was probably instrumental set-up problems such as plugged oxygen influx or different medium composition at the bottom of the feedstock bottle. The consortium composition could not be assessed due to bad sample quality. But a beginning system breakdown can be excluded as a reason, as the 4-CS was not above the toxicity concentration and did not increase further.

The added strain did not dominate the consortium nor did it substitute one of the consortium members or become washed out until the end of the experiment. *P.* sp. B13 SN45P established as new consortium member.

3.4.5 Summary: addition optimised degraders

The results of the single experiments are summarised in Table 12.

Table 12: Cell numbers and effects of competitor strains added to the consortium maintained with 4-CS or 4-CS and 4-CC in continuous culture

strain	**C-source**	**cell number competitor (cells ml^{-1})**			**abun-dance**	**wa/ st[b]**
		beginning	after x days	steady state[a]	steady state*	
A02	5 mM 4-CS	2.8×10^8	8.7×10^7 (15 d) 6.5×10^6 (60 d)	-	-	wa
G7::4/4	5 mM 4-CS	4×10^8	1×10^8 (60 d)	9×10^7	14%	st
G7::4/4	10 mM 4-CS	3×10^8	3.8×10^8 (8 d)	3.2×10^8	23%	st
B 13 SN 45P	5 mM 4-CS and 5 mM 4-CC	1×10^8	1.6×10^8 (15 d)	1.2×10^8	10%	st

[a] average; [b] wa = washout; st = steady state;

3.5 Influence of reduced oxygen concentration

The degradation of pollutants in the field (which can be soil or sediment in rivers) was found to be influenced by the oxygen concentration (Ghiorse & Wilson, 1988; Hopkins et al., 1993). Dissolved oxygen concentration regulates the synthesis and activity of the enzymes responsible for the initial attack on the aromatic ring of hydrocarbons (Villiesid & Lilly, 1992). The oxygen is required as cosubstrate for oxygenation reactions and as a terminal electron acceptor. As a result, reduced oxygen concentration may lead to the accumulation of toxic intermediates such as chlorocatechols (Fava et al., 1993). Therefore was the influence of oxygen reduction on the degradative performance of 4-CS degrading consortium studied.

Two 2.5 l chemostats were maintained in parallel as described in Materials and Methods 2.5.5.5. The air influx was controlled with gas-flow meters (see 2.5.5.7), to reduce the oxygen concentration to 10% saturation and below. The flux was 1 vvm (volume per volume and minute, e.g. 1 vvm = 2.5 l O_2/2.5 l reaction volume min^{-1}) in the undisturbed control. In the disturbed reactor, the flux was reduced to 0.1, to 0.01 and finally to 0.006 vvm, which resulted in oxygen concentrations of undisturbed 80% and disturbed 80%, 62% and 10%. In the latter case the concentration was fluctuating, and the lowest values reached were 8% and 2% saturation. The concentration of oxygen at 100% saturation in water at 1 bar and 12 °C is 10.8 (mg O_2) l^{-1} (Greenberg, 1965); therefore is the corresponding oxygen concentration at 10% , 8% and 2% saturation 1.1 (mg O_2) l^{-1}, 0.9 (mg O_2) l^{-1} and 0.2 (mg O_2) l^{-1}, respectively.

An overview of the development of the oxygen reduction experiment is plotted in Figure 51.

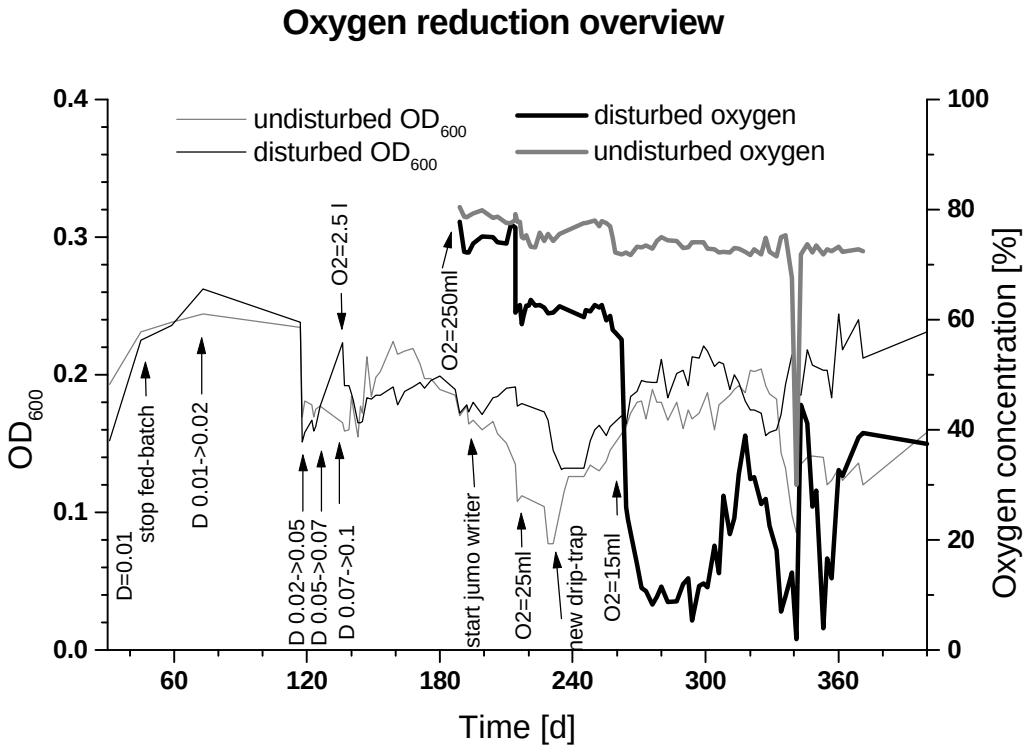

Figure 51: Overview of optical density (small lines) and oxygen concentration (big lines) of undisturbed -1 vvm oxygen supply - (grey graph) and disturbed -oxygen concentration reduced in steps to 0.006 vvm (black graph) chemostat, maintaining 4-chlorosalicylate degrading consortium;

The standard dilution rate of 0.1 d^{-1} was reached at day 140 of continuous culture (see Figure 51). The oxygen influx was fixed to 1 vvm, and after 30 days the steady state was reached. On day 185 the oxygen measurement begun, as the oxygen monitoring system was available. The first oxygen reduction was performed, and the air influx reduction resulted in a 5% lower oxygen tension in the disturbed chemostat. After another 30 days, on day 214, the influx was reduced to 25 ml min^{-1}, corresponding to 0.01 vvm. This resulted in a drop in oxygen concentration to about 62% saturation. At the same time the optical density decreased. However, this effect was not due to lowered oxygen concentration as the optical density decreased also in the control chemostat. The drip traps were overgrown and less medium reached the reaction vessel. The drip traps were exchanged at day 235, resulting in an increase of the cell density. After a recovery of the cell density, the oxygen influx in the disturbed chemostat was reduced again. The development of both chemostats from this point on is shown in detail in Figure 52, Figure 53 and Figure 54.

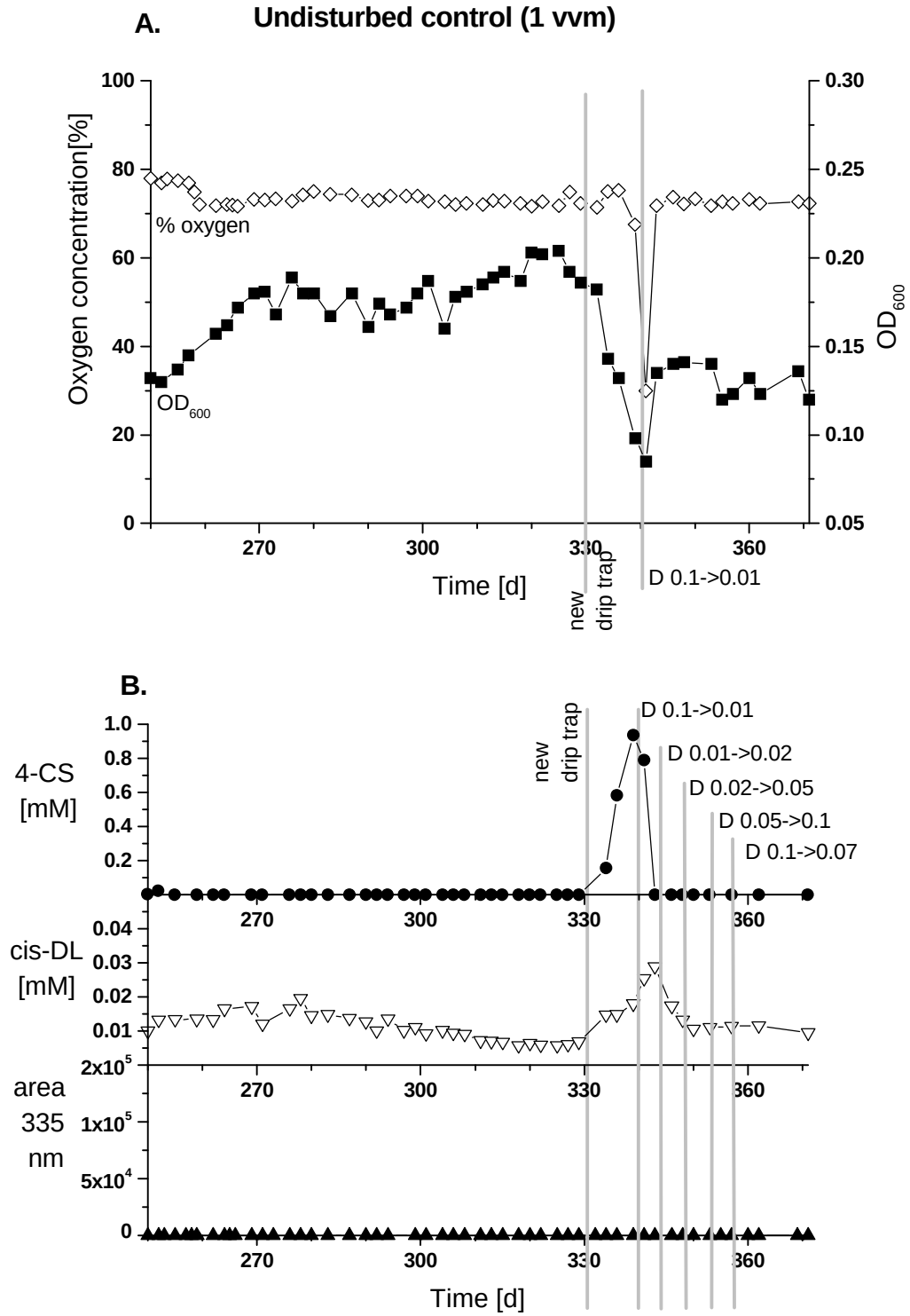

Figure 52: A. Oxygen concentration (◇) and optical density (■), B. 4-chlorosalicylate (●), *cis*-dienelactone (▽) concentration and area of unknown substance (▲) of undisturbed consortium growing with 1 vvm O_2 influx;

The oxygen concentration, the OD_{600}, the 4-CS and the *cis*-dienelactone concentration in the undisturbed reaction vessel remained mostly unchanged (see Figure 52). An exception is the time after day 330. On day 330 were the drip traps exchanged the second time. The cells could obviously not adapt their growth rate so fast to the now higher substrate concentration

in the influx. Therefore a washout of cells, as evidenced by a decline in OD_{600}, occurred between day 330 and day 340 (see Figure 52A) and 4-CS accumulated in the medium (see Figure 52B). Therefore, the dilution rate was decreased. Following, the accumulated 4-CS was consumed, which resulted in a short-time decrease of the oxygen concentration in the undisturbed chemostat between day 340 and day 342. The dilution rate was stepwise increased until the dilution rate 0.1 d^{-1} was reached. Finally it was reduced again to 0.07 d^{-1}. In the undisturbed chemostat, the accumulation of 4-CS due to the exchange of the drip trap could be observed. An increase of *cis*-dienelactone to 30 µM was observed at the same time. The development of the consortium composition of the undisturbed chemostat can be seen in Figure 54A.

The development of the chemostat disturbed by oxygen reduction is shown in Figure 53. The reduction of oxygen influx to 0.1 and 0.01 vvm, which resulted in an oxygen concentration of 80% and 60%, respectively, had no influence on the 4-CS degradation and no *cis*-dienelactone accumulated (see Figure 53B). The reduction of oxygen influx to 15 ml min^{-1}, corresponding to 0.006 vvm, resulted in a decrease of oxygen concentration in the reaction vessel to below 10% saturation between day 270 and day 300. No influence on the 4-CS concentration could be observed. The cell number increased minimally. However, the colour of the chemostat changed, turning yellow. When the cell-free extract was measured by HPLC, the occurrence of an unknown substance with the maximum wavelength of 335 nm was observed. The unknown substance is most likely a product of the *meta*-cleavage of chlorinated catechol. *Meta*-cleavage of 4-chlorocatechol results in the production of 5-chloro-2-hydroxymuconic semialdehyde. The maximum absorption spectrum of the yellow cell-free extract, which was observed under oxygen limitation in this experiment, was 379 nm under neutral conditions. The absorption spectrum of the yellow substance, when measured with HPLC in an acidic flow sheet, reduced to 335 nm. After day 300, an effect of cell growth in the drip trap was observed. Due to the cells consuming substrate in the drip trap less substrate reached the reaction vessel. The cell number decreased, and the oxygen concentration in the reaction vessel rose above 10%. The yellow colour vanished concomitant with the substance with the maximum wavelength of 335 nm. When the drip trap was exchanged, an accumulation of 4-CS to 0.2 mM was observed at day 340 (see Figure 53B). This concentration is lower than the amount of 4-CS accumulated as reaction to the drip trap exchange in the undisturbed chemostat. During the following course of lowering and raising the dilution rate, the oxygen concentration dropped two more times below 10%. Both times the occurrence of the unknown yellow substance was observed as a reaction to the low oxygen concentration.

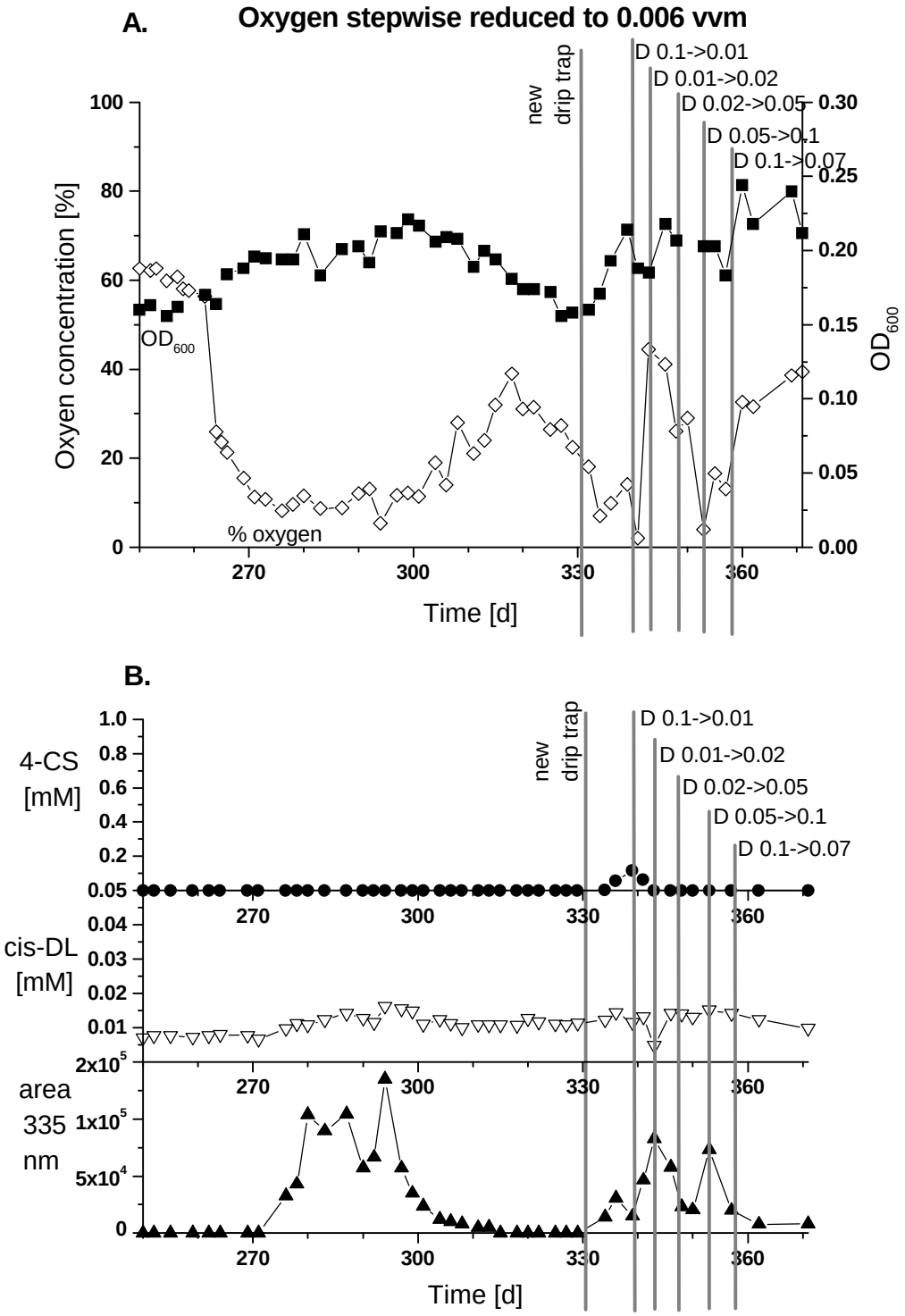

Figure 53: Oxygen concentration (◇) and optical density (■), B. 4-chlorosalicylate (●), *cis*-dienelactone (▽) concentration and area of unknown substance (▲) of consortium disturbed by stepwise oxygen reduction to 0.006 vvm;

The absolute abundance of consortium cells in the undisturbed experiment and the experiment disturbed by oxygen reduction are shown in Figure 54. Shifts in cell number can be observed. Under the influence of the disturbance (day 270 to day 306, and on day 340), the cell number was increasing in the disturbed chemostat. However, a look at the undisturbed chemostat

reveals that this cell number was also increasing during this time. What is observed here is the reaction of the consortium due to the "shock load" of 4-CS after the exchange of the drip trap.

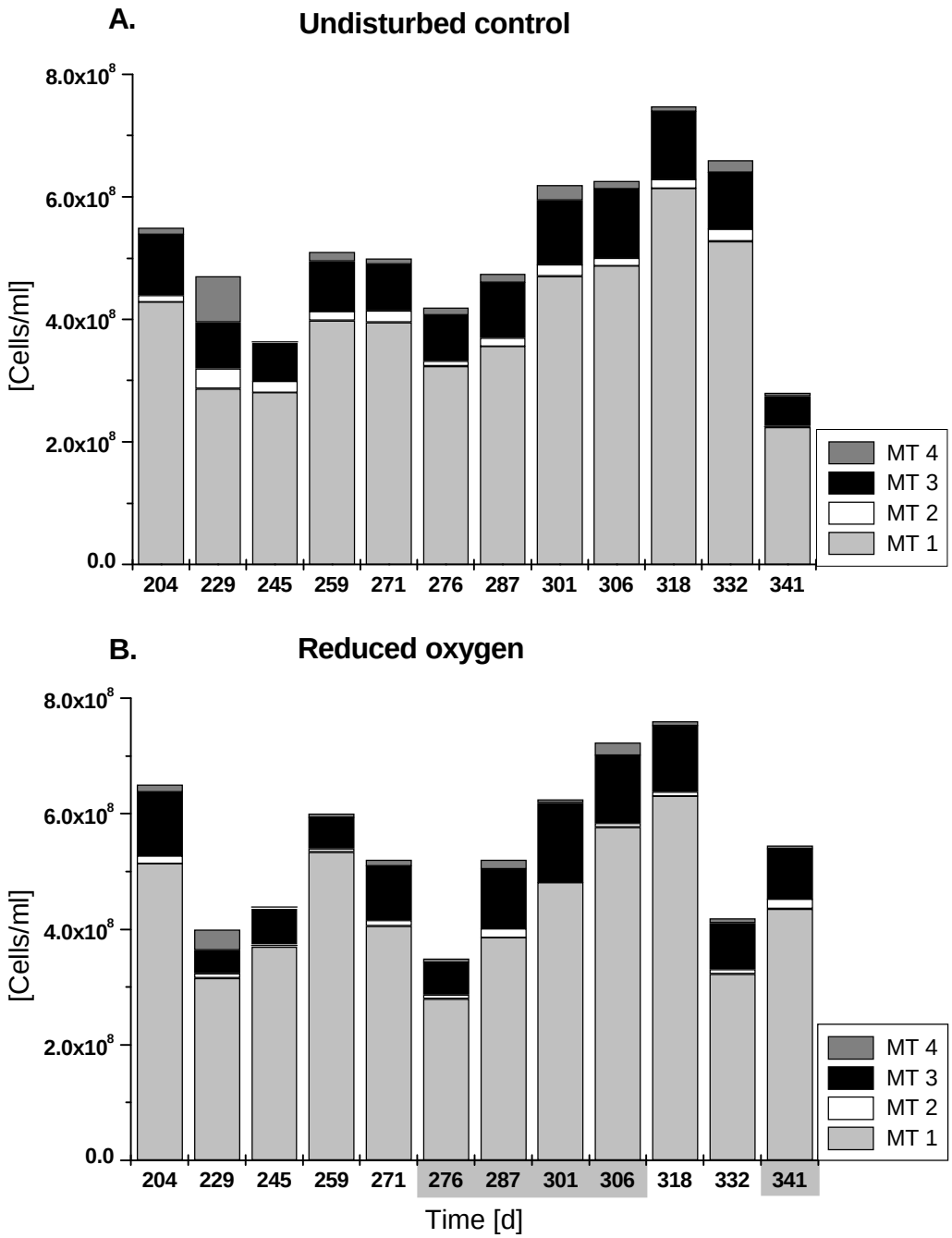

Figure 54: Absolute abundance of consortium members in undisturbed chemostat (oxygen influx 1 vvm) and chemostat disturbed by stepwise oxygen reduction to 0.006 vvm; grey areas indicate days when oxygen concentration dropped below 10% saturation.

In both chemostats the cell number increased between day 235 and day 320, because more carbon was available. The average consortium composition in the undisturbed chemostat was MT 1 = 77%, MT 2 = 3%, MT 3 = 17%, MT 4 = 4%; the average consortium composition in the disturbed chemostat at oxygen concentration levels above 10% was MT 1 = 81%, MT 2 = 1%, MT 3 = 14%, MT 4 = 3%; in the time span when the oxygen concentration dropped

below 10% the average consortium composition was MT 1 = 78%, MT 2 = 2%, MT 3 = 18%, MT 4 = 2%. It is apparent, that no significant changes in the consortium structure occurred during the influence of the disturbance.

3.5.1 Enzyme activity test

Raw extracts of consortium samples from the two chemostats from day 340, when the oxygen concentration of the disturbed chemostat was again below 10% and the yellow substance occurred, were tested for the occurrence of *meta*-cleavage enzymes (see Materials and Methods 2.7.2). To test for the activity of the specific enzymes, two parts of the raw extracts were treated differently, one to eliminate the activity of *meta*-cleaving enzymes, one to eliminate the activity of *ortho*-cleaving enzymes (see Materials and Methods 2.7.3).

Table 13: Specific activities of catabolic enzymes in cell extracts of undisturbed chemostat and chemostat disturbed by oxygen reduction below 10%, grown on 4-CS

Enzyme activity	**Assay substrate**	**Specific activity** (U g^{-1} protein) of cells of	
		(undisturbed)	**(reduced O_2)**
Catechol 1,2-Dioxygenase	Catechol	nd	1090 (100%)*
and	3-Chlorocatechol	nd	10 (1%)*
Catechol 2,3-Dioxygenase	4-Chlorocatechol	nd	285 (26%)*
Catechol 1,2-Dioxygenase	Catechol	780	1020 (94%)*
(0.01% H_2O_2) **(*ortho*)**	4-Chlorocatechol	130	450 (42%)*
Catechol 2,3-Dioxygenase	Catechol	3	6 (0.6%)*
(50 °C, 10 min) **(*meta*)**	4-Chlorocatechol	nd	**8 (1%)***

* Relative enzyme activities are expressed as percentages of unchlorinated catechols (= 100%); in brackets is the active enzyme cleavage; nd = not determined;

In Table 13, the enzyme activities of the ring-cleavage enzymes are displayed. A low but significant *meta*-cleavage in extracts of cells collected from the disturbed chemostat was observed. Both untreated raw extracts show different activity. The activity of *ortho*-cleavage enzymes of the undisturbed chemostat are as expected. Lower *meta*-cleavage enzyme activity was observed in the undisturbed chemostat compared to the disturbed chemostat. In the chemostat disturbed by oxygen reduction, the combined enzyme activities were similar to the values measured by Pelz (Pelz, 1999a). The author observed activity of (chloro-)catechol 1,2-dioxygenase with catechol as substrate 100% and with 4-chlorocatechol as substrate 45%.

4 Discussion

The aim of this thesis was the identification of factors which have an influence on the structure and function of a 4-chlorosalicylate degrading consortium and the study of reaction mechanisms displayed by the consortium. The background is that bioremediation in the field is not only hindered by the interaction between the degrading organism or the degrading community and the pollutant, but also the fitness of an inoculated strain or the performance of the community is influenced by the (always changing) environmental factors. The hypothesis is that consortia or communities have mechanisms to react to these changes in the environmental conditions, which allow them to continue their degrading activity. A 4-CS degrading consortium, maintained in continuous culture, was the subject of this study, as such complex issues can not be studied on communities in the field.

The build-up of a stable consortium structure under undisturbed conditions was necessary to be able to compare the influence of the disturbances upon consortium structure and function. This stability is based on interactions of the consortium members (the 4-CS degrading network, Chapter 1.4.5). It was found that these interactions are only displayed by enhanced degradation rates of the consortium compared to its primary degrader *P*. sp. MT 1. The similarity of the other kinetic parameters of *P*. sp. MT 1 and the consortium will be discussed first.

The undisturbed consortium under continuous culture conditions, the roles of the consortium members *A. xylosoxidans* MT 3 and *P*. sp. MT 4 and the observed colouration of the reaction liquid will be discussed next. These findings support the understanding of the consortium and are valuable for the interpretation of the consortium behaviour after applied disturbances. Reactions to disturbances can only be observed if the consortium can be maintained under steady-state conditions where the structure and function of the consortium do not change.

The reactions of the consortium to the different applied disturbances (mixed substrate utilisation, addition of optimised degraders and reduction of oxygen concentration) will be compared to other studies.

Finally the experimental set-up to study the influences upon the consortium structure and function will be discussed.

4.1 Kinetic parameters of the consortium and *Pseudomonas* sp. MT 1

In addition to the estimation of kinetic parameters for the consortium, which were needed for the planning and analysis of experiments, kinetic parameters were assessed for the primary degrader *P.* sp. MT 1. Batch cultures with different 4-CS concentrations inoculated with the consortium were performed and the maximal growth rate, the doubling time, the substrate

constant, the degradation rate and the yield characteristic kinetic parameters of the consortium and of *P*. sp. MT 1 were assessed.

4.1.1 Kinetic parameters of consortium

The mean specific growth rate of the consortium 0.018 h^{-1}. In one hour, 0.018 g new cells were generated from 1 g cells. This corresponds to a doubling time of 39 h. Easily degradable carbon sources such as glucose or propionic acids can be degraded by heterogeneous populations with doubling times of 1.4 h or 1.8 h, (growth rates of 0.49 or 0.38 h^{-1}), respectively (Pfeil & Gaudy, 1971). The lower growth rate of the consortium was certainly a result of the nature of the carbon source. The doubling times of consortia degrading carbon sources, having more complicated degradation, are longer. For example, the doubling time of a consortium degrading aromatic compounds (Solvesso 100) was t_d = 7-10 h (μ_{max} = 0.099-0.069 h^{-1}) (Stoffels et al., 1998), or methane sulfonic acid was t_d = 41 h (μ_{max} = 0.017 h^{-1}) (Magliette et al., 1996). These doubling times match very well with the doubling time of the consortium. Growth rates of pure cultures of *Pseudomonas* on 4-CS were previously estimated. Rubio found *Pseudomonas* WR 4016 had a generation time when grown with 4-CS in batch culture of 14 h (μ_{max} = 0.05 h^{-1}) (Rubio et al., 1986a). The author used higher optical densities (compared to this work) as inoculum $E_{546} > 0.25$.

The Monod substrate saturation constant (K_S), which equals the substrate concentration that supports a growth rate one half of the maximum, of the consortium acquired by the non-linear fitting of the degradation rates at different substrate concentrations (Figure 12) of the consortium is 0.367 mM (63 mg l^{-1}) for the consortium. Owens et al. summarised K_S values of various microbial strains with different substrates and observed extreme values, such as 110 µM or 2170 µM for *Streptococcus* and very low values 0.005 or 0.001 µM for *Escherichia coli* (Owens & Legan, 1987). Compared to Owens' data the K_S of the consortium is high. But it had been observed that the maximum growth rate of *Xanthobacter autotrophicus* GJ10 on a xenobioticum, 1,2-dichloroethane (with biotin supplementation), was 0.105 h^{-1} and the substrate saturation constant was 260 µM (van den Wijngaard et al., 1993). Although the K_S value for the consortium is high, it is apparent, that with xenobiotic substances values in this region can be reached.

The average degradation rate for all 4-CS concentrations was 0.364 mM h^{-1}(g dw)$^{-1}$ for the consortium (see Chapter 3.1.4).

The growth yields of the consortium appear to decrease with increasing substrate concentrations. With 1 mM 4-CS the consortium had a yield of 0.23. The growth yield of *Comamonas testosteroni* CPW301, degrading a resembling substrate, 4-chlorophenol (4-CP), had been observed to be about 0.11 (g dw) g^{-1} 4-CP, when the initial 4-CP concentration was less than 0.9 mM (Sung Bae et al., 1996). This strain utilised 4-CP via the *meta*-cleavage pathway. Although 4-CS is cleaved via the *ortho*-cleavage pathway, the observed yield of the consortium is in an expected range for these kinds of substrates. Beyond the 4-CS

concentration of 2 mM, very low yields were observed. 4-CS concentrations higher than 1 mM resulted in the occurrence of a brown colour. The brown colour results from the autooxidation of the catechols and is discussed in more detail in Chapter 4.2.2. The decrease of the growth rate and the yield at higher substrate concentrations indicate inhibition by toxic metabolites. The inhibitor is probably 4-chlorocatechol, which is a metabolite of 4-CS degradation. Chlorinated catechols and their autooxidation products are known to be toxic at high concentrations (for example Bartels et al. (1984)). The acquired data did unfortunately not allow to determine the inhibition constant. A large number of factors influence the biomass yield. These include the substrate and source of nitrogen, the composition of the medium, the pH and temperature and the presence of inhibitors. In this work it was not studied if different media or incubation temperatures would enhance the yield, because the results of this work should be comparable to previous work (Frech, 1996; Pelz, 1999a), which was performed under the same conditions. In these studies was the temperature chosen to match environmental conditions.

4.1.2 Kinetic parameters of *P.* sp. MT 1

The average growth rate with 1 mM 4-CS of *P.* sp. MT 1 was 0.019 h^{-1} (t_d = 39 h), and this value is below the values observed by *Pseudomonas* strains growing on 4-CS, which achieved growth rates of μ_{max} = 0.05 h^{-1} (t_d = 14 h) (Rubio et al., 1986a). Rubio's strain was able to grow with concentrations up to 5 mmol l^{-1} 4-CS. The same author was able to shorten the generation times by prolonged subcultivation of constructed strains to t_d = 7-12 h (μ_{max} = 0.099-0.058 h^{-1}) (Rubio et al., 1986b). Rubio et al. incubated the strains at 30 °C, the batch cultures of *P.* sp. MT 1 were incubated at the same temperature. On the other hand, in a continuous culture experiment performed by Pelz was the maximal growth rate of *P.* sp. MT 1 = 0.2 d^{-1}, this corresponds to 0.008 h^{-1} (Pelz, 1999a). This rate is lower as the growth rate aquired during this thesis for *P.* sp. MT 1.
The data used to calculate the maximum degradation rate could not be interpreted for the calculation of K_S for *P.* sp. MT 1.

4.1.3 Comparison of kinetic parameters of consortium to *P.* sp. MT 1

Often consortia (e.g. (De Souza et al., 1998; Feigel & Knackmuss, 1993)) display enhanced kinetic parameters compared to the primary degrader. The results of the analysis of batch cultures with varying 4-CS concentrations of the consortium and *P.* sp. MT 1 did show that the values for *P.* sp. MT 1 and the consortium were for the most part similar (Table 9), only the degradation rate of the consortium was 20% higher compared to the value of the primary degrader.

Generally, it is difficult to asses these values in batch cultures as it is necessary to work with very low substrate concentrations. Therefore, the here acquired data showed a high scatter. An

alternative way to assess the yield and the maintenance energy is to measure the substrate concentration and biomass formation of a continuous culture of *P*. sp. MT 1 and the consortium at different dilution rates. The data can be plotted, and the linear graph will show yield and maintenance energy, described by the equation: μ (growth rate) = Y_{XS} (Yield) *v*-Y_{XS} (maintenance energy). This method gives very good results, but could not be performed during this study. This method requires very low dilution rates and it takes a long time until the steady states are reached.

Under different cultivation conditions, namely continuous culture, differences in the growth behaviour of the consortium and the main degrader *P*. sp. MT 1 were previously observed (Pelz, 1999a). The same 4-CS degrading consortium could be maintained in continuous culture up to a dilution rate of 0.64 d^{-1}, whereas *P*. sp. MT 1 could only be maintained up to a dilution rate of 0.2 d^{-1}; then the metabolite protoanemonin occurred, which caused the cell death of *P*. sp. MT 1. In continuos culture under steady state conditions is the dilution rate the maximal growth rate. In Pelz' experiment was the maximal growth rate of the consortium 0.026 h^{-1}, this is higher as the value calculated in batch experiments in this thesis. The maximal growth rate of *P*. sp. MT 1 in Pelz' experiment was 0.008 h^{-1}. The occurrence of the toxic metabolite protoanemonin during *P*. sp. MT 1 growth on 4-CS with high dilution rates shows, that parameters different from the kinetic parameters influence the consortium cohesion. These parameters are the mineralisation of metabolites, which would otherwise become toxic for the consortium, by consortium members.

Caldwell demanded that the primary test of synergy (to verify a community) is that the cultured association proliferate and convert abiotic resources to biotic resources more effectively than its component members when cultivated individually (Caldwell et al., 1997). In the literature, the higher growth constants for consortia compared to pure cultures of their main degraders were observed, and examples are shown in Table 14. As in this study, the maximal growth rate of the orcinol degrading consortium did not differ from the maximal growth rate of the pure culture, but the K_S values did differ (Slater & Lovatt, 1984). The orcinol degrading consortium had a lower K_S value than the pure culture, indicating a higher affinity for the substrate. Another example of enhanced degradation of consortia compared to pure cultures of the main degrader is the above mentioned catabolism of atrazine, the consortium attained higher cell densities and removed atrazine better as the primary degrader *Clavibacter michiganese* (De Souza et al., 1998).

Table 14: Different growth parameters of consortia compared to pure cultures of one of their members

Reference	C source	pure culture	consortium
(Harrison, 1978)	methanol	μ_{max} = 0.16 h^{-1}	2 organisms: 0.19 h^{-1} all organisms: 0.64 h^{-1}
(Slater & Lovatt, 1984), page 453	orcinol (3,5-dihydroxy-toluene)	μ_{max} = 0.29 h^{-1} K_s = 100 mg l^{-1} K_i = 690 mg l^{-1}	μ_{max} = 0.28 h^{-1} K_s = 71 mg l^{-1} K_i = 1750 mg l^{-1}

The modification of growth parameters is only one type of interaction occurring in microbial communities. Additionally, there are other mechanisms leading to the development of consortia or communities, as described by Slater & Lovatt (1984). Examples of other interactions are the provision of co-factors or nutrients or the removal of toxic products. All these interactions might not result in better kinetic parameters, still the interactions of the consortium members result in the build up of a consortium. The removal of metabolites under stress conditions such as high dilution rates in chemostat culture, shows that the 4-CS degrading consortium has an advantage, which might not be observed by merely comparing kinetic parameters under batch culture conditions.

Rogers et al. also observed that the enhanced degradation activity of a consortium could not be described by the kinetic parameters of its single members. The authors found that a binary 1:1 culture of the strains *Pseudomonas putida* F1 and *Burkholderia* sp. JS150 were able to degrade the toluene-phenol mixture more rapidly than each pure culture alone (Rogers et al., 2000). Growth of the binary mixture on toluene or phenol alone resulted in significant interactions between the species. These interactions could not be described by a pure and simple competition model and were substrate dependent. The growth of one strain was inhibited, whereas the growth of the other strain was unaffected or enhanced. The production of one substance that inhibited and of another substance that enhanced the growth of the other strain was observed. Rogers et al. summarised that monoculture kinetic parameters were not sufficient to describe the mixed culture kinetics in any experiment, due to the observed interspecies interactions.

4.2 Further insights into 4-CS degrading consortium

4.2.1 Undisturbed composition and stability of consortium

Kinetic parameters of the consortium were measured (Chapter 4.1.1). Additionally the description of consortium composition and 4-CS degradation function under undisturbed conditions was assessed (Chapters 3.2.1 and 3.2.2). This set of information provided the basis to control the steady-state conditions of the consortium prior to application of the disturbances.

Two different average consortium composition were found (see Chapter 3.2.2), one for the batch cultures and one for the fed-batch and continuous culture, but the difference between the two consortium structures was small. In fed-batch and continuous culture, the abundance of *P*. sp. MT 1 and *A. xylosoxidans* MT 3 was 2% and 3% higher, respectively. The abundance of *P*. sp. MT 4 was 4% lower compared to the batch culture results. Two explanations for this result are possible. First, the culturing method could have an influence. A variation can occur, as the endpoint of the exponential growth phase in batch culture is difficult to assess. A second reason could be that the experiments were performed at different inoculation temperatures. The continuous culture for the oxygen reduction experiment was performed at 12 °C, the other continuous culture experiments and the fed-batch experiment were performed at room temperature at 20-22 °C, and the batch experiments were performed at 30 °C. It is possible that the strain *P*. sp. MT 4 has better growth abilities at higher temperatures. The temperature has an influence upon the growth rate and the yield of microbial strains ((Atkinson & Mavituna, 1991), Chapter 4, page 148). The temperature will surely influence the growth rates of the single consortium members differently and therefore result in a different consortium structure. Influence of the incubation temperature on the composition of an archeal community was found previously (Fey & Conrad, 2000). The authors could show that temperature affected the carbon and electron flow in methanogenic rice soil and affected the composition of the archeal community. This example shows that it is possible that the consortium structure could be influenced by the different temperatures. Therefore it is also possible that the different consortium compositions, which were observed in batch or fed-batch and continuous culture, are based on the different incubation temperatures.

The consortium composition found in this work can be compared to the results of earlier studies of the consortium. Frech and Pelz observed a consortium composition of the 4-CS degrading consortium in continuous culture at 12 °C of MT 1 = 84 ± 2.7%, MT 2 = 0.7 ± 0.3%, MT 3 = 7.5 ± 3.8% and MT 4 = 8 ± 4.2% (Frech, 1996; Pelz et al., 1999b). This consortium composition of the undisturbed consortium differed from the afore mentioned values. In the studies of Frech and Pelz et al., the abundance of *P*. sp. MT 1 was 8% and of *P*. sp. MT 4 was 5% higher, the abundance of *A. xylosoxidans* MT 3 was 11% lower compared to the undisturbed consortium composition shown above. Both workers used 2.5 l chemostats with 1 l working volume, which ran for at least 3 months at 12 °C. The observed differences could be the result of an "ageing" of the consortium under prolonged continuous cultivation. The small difference in abundance of *P*. sp. MT 1 and *P*. sp. MT 4 probably had no large influence upon the consortium function. *A. xylosoxidans* MT 3 plays an important role in the consortium, therefore the lower abundance could result in a reduced degrading capacity. This was not observed. The consortium structure of the studies of Frech and Pelz et al. was assessed by different persons and some of the differences could result from the different person performing the counting.

Caldwell et al. (1997) summarised characteristics of microbial communities. To confirm that a community culture has been obtained, it is important to demonstrate that the association of organisms meets, among others, the criterion of autopoiesis. It could be shown that autopoiesis occurs, as the consortium can be re-associated from pure cultures of the single consortium member strains. An interesting observation is that even under unfavourable start conditions, e.g. the occurrence of a contaminant in the fed-batch control experiment (Figure 17), the expected undisturbed consortium composition starts to build up under the influence of the continuous 4-CS feeding. Stability of the consortium composition was achieved if the maintenance time of the continuous culture was kept below 6 months. Longer culture periods occasionally resulted in a different consortium composition, dominated by *P.* sp. MT 1 (and in the occurrence of contaminating species). Additionally, mechanical problems such as the plugging of drip traps could be avoided, keeping the culturing time short. The reduction of the time until a steady-state condition was reached meets this criterion and was only possible due to the ability to control whether the required steady-state consortium composition was achieved.

4.2.2 Brown colouration of consortium medium

Batch culture growth with 4-CS concentration of 2 mM or higher resulted in a brown colouration of the medium (see Chapter 3.1.2). The "shock load" of 10 mM 4-chlorocatechol in continuous culture resulted in a system breakdown. The system breakdown corresponded with the development of a brown colour which remained in the medium.

The production of brown colours following the degradation of chloroaromatics via 3-chlorocatechol using the *meta*-cleavage pathway has been widely reported in the literature (Adams et al., 1992; Klemba et al., 2000). Colouration of the medium is due to the build up of 3-chlorocatechol (or 4-chlorocatechol), which polymerises due to auto-oxidation. The polymerisation process from chlorocatechols results in a drop in chlorocatechol concentrations measured by HPLC. The drop in chlorocatechol concentration is usually not due to degradation of the catechols, but due to the polymerisation process. This can be evaluated by control experiments containing chlorocatechols in the absence of any microbial inoculum and incubation in parallel. An increase in the optical density at 600 nm due to the development of extracellular pigment production following degradation of 3-chlorobenzoate has been described in the literature (Fava et al., 1993). Although the auto-oxidation of chlorinated catechols is often described for 3-chlorocatechol, it is also valid for 4-chlorocatechol. Development of pigment production resulting from polymerisation of 4-chlorocatechol was previously observed (Haller & Finn, 1979; Wieser et al., 1994). Further problems generating from interference of the brown colour with optical density measurements are discussed by Farrell & Quilty (1999). 4-CC is oxidised by means of an unknown reaction. The product, 4-chloro-*ortho*-chinone, is substrate for a radical polymerisation. It has been

suggested that polymerisation might not be based on auto-oxidation alone, but is a result of the activity of peroxidase and polyphenol oxidase enzymes (Fava et al., 1993).

4.2.3 Roles of consortium member *A. xylosoxidans* MT 3 and *P.* sp. MT 4

More information about the roles of consortium members *Achromobacter xylosoxidans* (*A. xylosoxidans*) MT 3 and *P.* sp. MT 4 was gained by study of the consortium structure. The strains *A. xylosoxidans* MT 3 and *P.* sp. MT 4 consume metabolites of the degradation pathway of *P.* sp. MT 1, thus preventing the whole system from toxification. This can be seen by the change in the abundance of the four consortium members during the first phase of batch growth (Figure 9B). The increase of strain *A. xylosoxidans* MT 3 at the beginning of the exponential growth phase was relevant for the consortium. Two growth rates were observed during consortium growth on 4-CS in batch culture (Figure 6B, Chapter 2.6.4). The first growth rate, not corresponding to 4-CS decrease, shows probably the growth of *A. xylosoxidans* MT 3 at a rate of approximately 0.04 h^{-1}. These first growth rate was observed in almost all batch culture experiments. It was repeatedly observed in this thesis that this increase in the abundance of *A. xylosoxidans* MT 3 was very necessary for the healthy development of the consortium. The strain *A. xylosoxidans* MT 3 possesses the chlorocatechol 1,2-dioxygenase. The occurrence of this enzyme is necessary for the whole the degradation ability of the consortium.
Lister et al. observed a comparable change in the consortium structure of a consortium, which consisted of two members during batch culture (Lister et al., 1996). During the growth phase of cocaine degradation, the *P. fluorescens* population had a higher proportion than the *Comamonas acidovorans* strain.

The simultaneous feeding of 5 mM 4-CC and 5 mM 4-CS resulted in an increase in the abundance of consortium member *A. xylosoxidans* MT 3 (see Chapter 3.4.4). Simultaneous degradation occurred. The colouration of the medium due to 4-CC accumulation and condensation as a result of higher 4-CC concentration are discussed above in Chapter 4.2.2.
This increase in abundance of *A. xylosoxidans* MT 3 was expected, as the strain contains chlorocatechol cleavage enzymes and has certainly the highest affinity for 4-CC compared to *P.* sp. MT 1 and *P.* sp. MT 4. As described in Chapter 1.4.5, 4-CC is a metabolite of 4-CS degradation. The substrate can also be degraded by *P.* sp. MT 1 and *P.* sp. MT 4, but these strains contain only the enzyme catechol 1,2-dioxygenase. This enzyme differs from chlorocatechol 1,2-dioxygenases by its broader substrate specificity and low 3- and 4-chlorocatechol cleavage activity. *A. xylosoxidans* MT 3 contains the chlorocatechol 1,2-dioxygenase, which has specific ability of chlorinated catechol degradation, this explains the increase of *A. xylosoxidans* MT 3.
Pelz observed that pure cultures of *P.* sp. MT 1, *A. xylosoxidans* MT 3 and *P.* sp. MT 4 grown with 2 mM 4-CC and 0.2 g l^{-1} yeast extract incorporated ^{13}C labelled 4-CC. This indicates that

all three strains can use 4-CC as carbon source. Pelz also performed a pulse feed of ^{13}C labelled 4-CC to the same 4-CS degrading consortium studied in this thesis growing in continuous culture. The immunocapture analysis of the single consortium members showed that 4-CC incorporation in *A. xylosoxidans* MT 3 after 2.5 h and 4-CC incorporation in *P.* sp. MT 1 and *P.* sp. MT 4 after 6 h. This observation shows that *A. xylosoxidans* MT 3 probably has the highest affinity for the substrate. This observation matched with the findings of the here discussed experiment, and indicates that *A. xylosoxidans* MT 3 outcompeted the other strains and used 4-CC as carbon source.
4-CC was fed in the experiment displayed in Chapter 3.4.4 in the same amount as 4-CS. Therefore a consortium structure shift in which the second primary degrader would become 50% in abundance would be expected (e.g. the additional feeding of $^{1}/_{10}$ NB resulted in the increase of *E. brevis* MT 2 to 50% in abundance (see Chapter 3.3.2)). A lower abundance of only 30% was observed for *A. xylosoxidans* MT 3. This is due to the nature of the carbon source. The 4-CC condensated in the medium reservoir, as was indicated by a brown colouration, and therefore less than 5 mM 4-CC was available for cell utilisation.

The strain *P.* sp. MT 4 increased in abundance when the carbon source was omitted from the feedstock in fed-batch culture. This result indicates that the strain (together with *E. brevis* MT 2) is able to metabolise and grow cell debris from dying consortium cells. Probably has *P.* sp. MT 4 the highest affinity to this kind of complex carbon source. The strain is also able to use many different carbon sources. In a BIOLOG™ test, performed by Pelz (personal communication), *P.* sp. MT 4 was able to use 59 of the 95 carbon sources, whereas *E. brevis* MT 2 could only utilise 20 carbon sources. The ability to utilise cell debris as a carbon source should be kept in mind when the role of *P.* sp. MT 4 in further experiments is discussed. For example, in the batch experiments, when all carbon sources were used up, the cells started to die. An increase of *P.* sp. MT 4 in this phase may indicate that the strain feeds on dead cells, and the increase might not be a reaction to the disturbance. This was observed in batch experiments. Because of this observation, the yields were calculated and the consortium structure was noted at the end of the exponential phase in the batch experiment and not at the end of the experiment.

4.3 Reaction to disturbance by a second carbon source

In ecosystem habitats, both the rate and the extent of biodegradation of xenobiotic organic compounds are influenced by the presence of other organic compounds (Egli, 1995). It is difficult to study the influence of this factor upon communities in the field; therefore mixed substrate utilisation was studied by continuous culture of the 4-CS degrading consortium.

4.3.1 Summary and possible reasons for observed effects of additional ethanol, iso-propanol, histidine and $^{1}/_{10}$ NB feeding in continuous culture

The consortium was maintained with 5 mM 4-CS as carbon source in continuous culture and stressed by simultaneous feeding of ethanol, iso-propanol, histidine and $^{1}/_{10}$ NB, respectively. Good correlation between predicted (based on pre-experiments) and assessed values of consortium structure and influence upon the consortium function were observed (see 3.3.2, Table 10). It is not possible with the available amount of data to completely explain the reasons for the system breakdowns and simultaneous degradations, but some reasons can be excluded and possible solutions proposed.

- The additional ethanol and iso-propanol feeding led to an increase of *P*. sp. MT 4, and after 4 (ethanol) to 7 (iso-propanol) days the degradation of 4-CS stopped. The alcohols were still degraded and a washout of all the cells occurred. Because the concentration of 4-CS in the reaction vessel exceeded the inhibitory concentration of 2 mM, the breakdown continued along with the washout of the cells.

This result was expected due to the result of the pre-experiment (Table 10). Some reasons which could explain the breakdown of the system can be omitted. Deficiency of O_2, or competition for limiting nutrients can be excluded, due to the nature of the continuous culture system. O_2 should not be in the limiting range because the experiments in Chapter 3.5 showed that the oxygen influx under undisturbed conditions is sufficient and can be reduced dramatically (to 0.01 vvm) without resulting in a critical oxygen concentration. The medium contained sufficient nutrients to provide for simultaneous degradation of 4-CS along with a second carbon source, e.g., histidine (see Chapter 3.3.2).

In high concentrations (70% (v/v)) ethanol is used as a disinfectant, and the toxicity is based on the agglutination of proteins. Such a toxic effect of the ethanol can be excluded, as the concentration of ethanol in the experiment was 0.002% (v/v)) (15 mM). No toxic metabolites like protoanemonin, generating from incomplete 4-CS degradation by *P*. sp. MT 1, could be detected. A possible explanation might be that the other strains provide a better kinetic situation for *P*. sp. MT 1 because they take metabolites (4-chlorocatechol and protoanemonin) out of the degradation pathway of *P*. sp. MT 1 (see Chapter 1.4.5). When the second carbon source ethanol was applied, *P*. sp. MT 4 (and after 2 days also *A. xylosoxidans* MT 3) can use this carbon source (see pure culture studies Chapter 3.3.1.1). A catabolite repression in these strains could cause them to stop the degradation of the metabolites. The kinetic situation for *P*. sp. MT 1 would be more unfavourable and could create a situation in which *P*. sp. MT 1 was no longer able to degrade all available 4-CS, and the accumulation of the metabolites should be observed, but this was not the case. A possible explanation is that the concentration of the ethanol is not high enough to toxify the *P*. sp. MT 1 cells, but that the ethanol has an

inhibitory effect upon the main enzymes of the 4-CS degradation pathway, or that the uptake of the 4-CS is somehow hindered, as 4-CS started to accumulate in the reaction vessel.
The consortium composition changed, *P.* sp. MT 4 increased to over 50% in abundance. *P.* sp. MT 4 was able to degrade ethanol instantaneously (see Chapter 3.3.1.1). Therefore was the increase in cell number of this strain based on ethanol metabolisation. It is possible that the strain did additionally feed on the dying cells of *P.* sp. MT 1 and *A. xylosoxidans* MT 3, which were washed out (Chapter 4.2.3).

A similar result of the influence of ethanol added to a consortium degrading a polyvinyl alcohol (PVA) in continuous culture was previously observed. Shimao et al. observed different effects of additional ethanol feeding on a mixed culture of *P. putida* VM15A and *P.* sp. strain VM15C degrading 2.5 g l^{-1} (50 mM) PVA in continuous culture (Shimao et al., 1985). The additional feeding of ethanol resulted in the increase of PVA degradation and increases in the VM15C population. This enhanced PVA degradation was based on the provision of the growth factor pyrroloquinoline quinone by strain VM15A. When ethanol was fed at 0.1 g l^{-1} (2 mM), the cell concentration increased, but the consortium structure (98% VM15C and 2% VM15A) remained unchanged. Additional feeding of 1 g l^{-1} ethanol (20 mM) resulted in the decrease of the PVA degradation, compared to the 0.1 g l^{-1} additional ethanol feeding, but the degradation was still more apparent than without ethanol addition. The consortium structure shifted to (88% VM15C and 12% VM15A). Here the same tendency as in the ethanol addition experiment was apparent, the secondary or minor consumer increased in abundance. The reason for the decrease of the PVA degradation and VM15C concentration with increased ethanol concentration was unknown.

- Additional histidine feeding also led to an increase of *P.* sp. MT 4, and both carbon sources were degraded in parallel. After the end of the disturbance the system was able to recover.

These results matched with the results from the batch pre-experiment (Table 10). In pure cultures experiments with only one carbon source (Chapter 3.3.1.1), *P.* sp. MT 1, *P.* sp. MT 4 and *A. xylosoxidans* MT 3 were able to use histidine as a carbon source. After 2 days all three strains grew on the carbon source, but *P.* sp. MT 1 had the highest yield and was able to degrade histidine fastest, followed by *P.* sp. MT 4. As no catabolite repression was observed in batch culture for *P.* sp. MT 1 (see Chapter 3.3.1.2, Figure 23), this was also not expected for the continuous culture. Indeed, *P.* sp. MT 1 continued to degrade 4-CS. The average cell number of *P.* sp. MT 1 in continuous culture before day 7 and after day 22 was 4.8×10^8 cells ml^{-1}, the average cell number of *P.* sp. MT 1 between day 13 and day 19, when the additional histidine feeding was applied, was 1×10^9 cells ml^{-1}. This value is higher and indicates growth of *P.* sp. MT 1 cells supported by the carbon source histidine. It is possible that *P.* sp. MT 1 could degrade both carbon sources simultaneously (Simultaneous degradation of two

carbon sources by one *Pseudomonas* strain was previously described in the literature (Hutchinson & Robinson, 1988; Lendenmann et al., 1996; Reber & Kaiser, 1981)).
P. sp. MT 4 became the second most abundant strain in the additional histidine feeding phase. It is possible that *P*. sp. MT 4 had a higher affinity for histidine and could use most of the histidine as a carbon source. The community composition changed in that approximately 50% more biomass was produced after the start of the additional feeding of histidine. Most of this biomass was *P*. sp. MT 4 cells, but the concentration of *A. xylosoxidans* MT 3 also increased. If catabolite repression of *P*. sp. MT 4 or *A. xylosoxidans* MT 3 would occur, accumulation of metabolites would result along with a system breakdown. It was observed that this did not happen.

- Additional feeding of $^{1}/_{10}$ nutrient broth (NB) led to an increase of consortium member *E. brevis* MT 2. Both carbon sources were degraded in parallel, and an increase in the DOC indicated that either not all NB was degraded or that a metabolite (from NB substrate) accumulated in the reaction vessel. When the additional feeding of $^{1}/_{10}$ NB was stopped, a system breakdown was observed. This reaction is surprising, as a negative influence should have had an earlier effect. 4-CS was still degraded up to this point, and there was no apparent reason why the 4-CS degradation should not continue. Due to a disturbance of the HPLC measurement it could not be monitored, whether toxic metabolites of 4-CS degradation did occur. No changes in the colour of the medium indicating accumulation of 4-CC were observed.

The consortium was fed with $^{1}/_{10}$ NB as the only carbon source in a fed-batch pre-experiment (3.3.1.3, Figure 28). The same consortium structure shift as in the continuous culture experiment occurred, and *E. brevis* MT 2 increased. The relative abundance of the consortium members could be measured, but not the absolute abundance, due to the invasion of a foreign strain. In the fed-batch experiment, $^{1}/_{10}$ NB was used as the sole source of carbon and energy and differs in this way from the continuous culture experiment, in which the $^{1}/_{10}$ NB was fed in addition to 4-CS. But the fed-batch experiments can be compared to a continuous culture experiment performed by Frech with the same 4-CS degrading consortium (Frech, 1996). The substitution of 4-CS by $^{1}/_{10}$ NB for 14 days led to a shift in the consortium structure (see Table 10), and a similar increase of *E. brevis* MT 2 was observed. In the continuous culture experiment of this thesis, where 4-CS was still present, a similar increase of *E. brevis* MT 2 was observed under the influence of additional $^{1}/_{10}$ NB feeding (Table 10), but *P*. sp. MT 1 had a higher abundance and *P*. sp. MT 4 a lower abundance compared to Frech´s substitution experiment. This was expected because *P*. sp. MT 1 is the primary degrader of 4-CS.
The results from pure culture studies were sufficient to predict which strain would become the second primary degrader in the $^{1}/_{10}$ NB feeding experiment. Although all strains were able to use NB as a carbon source, *E. brevis* MT 2 had the highest yield and was able to degrade $^{1}/_{10}$ NB fastest. When $^{1}/_{10}$ NB was added, the strain could outcompete the other strains and

became a second primary degrader and *P*. sp. MT 1 and the other strains continued to degrade 4-CS.
Compared to the experiments were ethanol, iso-propanol and histidine were added and *P*. sp. MT 4 increased in abundance, here *E. brevis* MT 2 became the second primary degrader. But *E. brevis* MT 2 played no role in the degradation of 4-CS. It is assumed that the strain lived on cell debris. Therefore the omission of this strain in the consortium structure could not have caused the system breakdown.

The following possible explanations can be excluded:
The concentration of *P*. sp. MT 1 of 3.5×10^8 cells ml^{-1} at the end of the additional feeding of $^1/_{10}$ NB was too high to facilitate system breakdown due to low main degrader concentration. In Frech's experiment the concentration of *P*. sp. MT 1 at the end of the substitution phase was even lower, 3.5×10^7 cells ml^{-1}, and the system was able to recover.
The nature of the carbon source provides another possible explanation for the observed breakdown. It is possible that the complex carbon source NB provides some precursors for *P*. sp. MT 1, which *P*. sp. MT 1 has to produce itself after the stop of the additional feeding. Until this production is induced and started the 4-CS concentration could have accumulated to an inhibiting concentration. Similarly the induction of enzymes necessary for the degradation of the pollutant is important. Additional carbon sources can be useful for enzyme synthesis under transient conditions (Egli, 1995). The latter point works under the supposition that the availability of an alternative carbon/energy source helps the cell to avoid excess degradation of intracellular resources during adaptation to new growth conditions. But 4-CS was accumulated to 1 mM in about 7 days, and this time span is large enough to allow for *P*. sp. MT 1 to adjust to the new situation.
It is also possible that after an adaptation phase *A. xylosoxidans* MT 3 stopped performing its role in the 4-CS degradation process, which is to metabolise 4-CC. But this effect should have been observed directly after start of the additional $^1/_{10}$ NB feeding, because in the pure culture studies no lag phase was observed, when *A. xylosoxidans* MT 3 did grow on NB. The failure of metabolite degradation of *A. xylosoxidans* MT 3 should be apparent by an accumulation of 4-CC, which creates a brown colour in the medium. This colouration was not observed.

Possible explanations are:
E. brevis MT 2 could misroute 4-CS to the production of a toxic metabolite. This effect would only be apparent after the large increase in *E. brevis* MT 2 cell number after the additional $^1/_{10}$ NB feeding. It is known that *E. brevis* MT 2 can not use 4-CS as a carbon source. But it is not known, and can therefore not be excluded, if *E. brevis* MT 2 is able to co-metabolise or somehow transform 4-CS.
The increase in the DOC after addition of $^1/_{10}$ NB does not indicate the accumulation of a toxic metabolite, which could cause an inhibition of *P*. sp. MT 1, because the effect of this

inhibition would be observed earlier, after day 53. The unknown substance accumulated between day 37 and day 53, but between day 53 and day 65 the level of the substrate remained stable, as the steady state had built up. The main part of the metabolite (or indigestible part) did arose from the $^{1}/_{10}$ NB, as an increase in the DOC concentration to 80 (mg C) l^{-1} was observed in the fed-batch pre-experiment (Figure 28), in this experiment $^{1}/_{10}$ NB was the only carbon source. But the DOC in the continuous culture $^{1}/_{10}$ NB addition experiment increased to 100 (mg C) l^{-1}. It is highly probable that the major part is from the NB as in the pre-experiment. Probably the value is the summ of the background DOC of the $^{1}/_{10}$ NB and the 5 mM 4-CS experiments. Unfortunately the HPLC measurement was disturbed, so it could not be monitored to ascertain whether toxic metabolites accumulated in addition.

4.3.2 Reaction mechanisms of consortium to disturbance by additional feeding of easily utilised carbon sources

Table 15: Effects of the additional feeding of easily utilised carbon sources and 4-CC upon the 4-CS degrading consortium maintained in continuous culture

2nd C source	degr. 4-CS	degr. 2nd C-source	sim. degr./ washout	cons. member increase	system able to recover
ethanol	-	+	washout	MT 4	-
iso-propanol	-	+	washout	MT 4	-
histidine	+	+	sim. degr.	MT 4 (MT 1+ MT 3)	+
4-CC*	+	+	sim. degr.	MT 3	**?**
$^{1}/_{10}$ NB	+	+	sim. degr.	MT 2	-

*see Chapter 3.4.4

The additional feeding of easily utilised carbon sources always resulted in a change in the consortium structure. The results of additional feeding of easily utilised carbon sources are summarised in Table 15. The carbon sources histidine, $^{1}/_{10}$ NB and 4-CC were simultaneously degraded. These experiments show that the consortium had the ability to react to the disturbance and to continue to degrade 4-CS. In these cases, the consortium could retain its function because a secondary degrader changed to a second primary degrader. The limits of the efficiency of the system were reached by the additional feeding of ethanol and iso-propanol, and this caused the stop of the 4-CS degradation, and a system breakdown and washout resulted.

An interesting observation comparing the results of the mixed substrate utilisation experiments displayed in Table 15 is that a comparable consortium structure shift, the increase in abundance of consortium member *P*. sp. MT 4, was on one hand accompanied in the case of alcohol addition by a system breakdown, and on the other hand when histidine was added, simultaneous degradation occurred. This shows that the study of only structural change of a consortium is not efficient to predict the influence upon the function. The influence of both disturbances upon the degrader of the second substrate, *P*. sp. MT 4, was the same, this strain could degrade the alcohols and histidine, but the influence upon *P*. sp. MT 1 was different. On one hand, *P*. sp. MT 1 was inhibited by the alcohol, but on the other hand, the growth of *P*. sp. MT 1 was not hindered but enhanced by histidine.

4.3.2.1 Simultaneous degradation: successful cooperation of consortium members

In this work, the consortium was able to react to the disturbance, which was feeding of an additional easily utilised carbon source, because at least one of the consortium members degraded the second carbon source, while *P*. sp. MT 1 and the other consortium members continued to degrade the 4-CS.

The findings of the histidine, 4-CC and $^{1}/_{10}$ NB addition experiments concur with the generally accepted theorem, "that for stable coexistence to occur between primary consumers competing for nutrients, the number of these nutrients must be greater than or equal to the number of competing species" (Philips, 1973). Simultaneous degradation of mixed substrates by two primary degraders was shown before. Chian and Mateles showed repeatedly that when mixtures of glucose and butyrate were used, mixed cultures of two dominant species, each metabolising only one substrate, were obtained (Chian & Mateles, 1968).

The nature of the carbon source plays an important role on the outcome of experiments with the feeding of additional carbon sources (Schmidt & Alexander, 1985). Egli analysed that in ecosystems, both the rate and the extent of biodegradation of xenobiotic organic compounds at low concentrations are probably controlled by the presence of other organic compounds, and that the patterns of stimulation or inhibition of the degradation of pollutants observed at a particular time are to a large part a result of the particular mixture, concentration and availability of additional (carbon) compounds of natural origin (Egli, 1995). The findings of this study support this theory as the results of the experiments were mainly influenced by the nature of the second carbon source and the ability of the single consortium members to use this carbon source. The mixture and concentration of both carbon sources is also of importance; e.g. Babu studied simultaneous degradation of 3-chlorobenzoate (3-CBA) and phenolic compounds by a defined mixed culture and found that 3-CBA degradation was only complete when 3-CBA was equal in amount to or less than phenol (Babu et al., 1995).

The results of the mixed substrate study concur very well with the conclusions drawn by Ford when studying the effects of chemical stress on aquatic species composition and community structure (Ford, 1989). His general summary of the effects of chemical stress is here applied to the two observed reactions, once to the consortium structure change which resulted in the continuation of the function and once to the system breakdown. Contrary to Ford's observation, that the earliest phase of ecosystem response begins with the loss of species, resulted the additional feeding of histidine, 4-CC and $^{1}/_{10}$ NB not in the loss of species. This is a major advantage. The next step is that native species respond in individualistic ways, compositional changes were observed, and either *P.* sp. MT 4, *A. xylosoxidans* MT 3 or *E. brevis* MT 2 increased in abundance. No decline in species richness was observed. Ford states that the initial response of most aquatic ecosystems to chemical stress involves loss of sensitive species accompanied by changes in relative abundance of rapidly reproducing taxa. Although no loss was observed, the consortium structure change did occur. Whether this structural change has an effect upon the function depends on the structural organisation of the receiving system. Redundancy buffers the effects of species loss on functional parameters and significant changes of species composition can occur without affecting community function. This was observed. *E. brevis* MT 2 did increase in abundance (due to additional $^{1}/_{10}$ NB feeding) and the 4-CS degradation continued. *A. xylosoxidans* MT 3 and *P.* sp. MT 4 are secondary degraders of metabolites emitted from the 4-CS degradation by *P.* sp. MT 1. But neither the increase in abundance of *A. xylosoxidans* MT 3 (due to additional 4-CC feeding) nor the increase of *P.* sp. MT 4 (due to additional histidine feeding) resulted in the accumulation of metabolites. It is possible that the strains degraded the metabolites and the second carbon source similarly, or that the role of one consortium member was with a dilution rate of $D = 0.1\ d^{-1}$ not as important as with higher dilution rates. It was previously observed, that simultaneous degradation of two substrates occurred more easily at low dilution rates and/or low concentrations (Egli, 1995). The development of the response of the 4-CS degrading consortium concurs with known reactions to chemical stress.

The influence of redundancy upon consortium stability was also observed by Chapin, who states: "The apparent conflict between the perspectives that each species is important and that there is ecological redundancy among species is resolved when biotic composition is considered in terms of functional types of organisms and their environmental responses. Changes in the abundance of species that differ in ecosystem consequences should affect process rates or patterns, whereas the abundance of species with similar ecological effects should give stability to ecosystems" (Chapin III et al., 1997).
A similar observation was made by Hashsham et al. (2000). Studying methanogenic bioreactor communities perturbed by glucose, the authors observed that parallel substrate processing (in this case a network of multiple routes for substrate flow) resulted in a functionally more stable consortium than serial substrate processing (Hashsham et al., 2000).

In the 4-CS degrading consortium is *P.* sp. MT 1 the only strain able to transform 4-CS to 4-CC, but multiple routes for the metabolisation of 4-CC, namely the oxoadipate and the chlorocatechol pathway, exist.

A further factor influencing the response ability of the affected consortium to the chemical stress is the assortment of species. For example, Rogers et al. found that the rate and extent of biodegradation of toluene and phenol by a binary culture was influenced by the nature of the occurring interspecies interactions, such as inhibition or enhancement of consortium member growth (Rogers et al., 2000).

Simultaneous degradation of phenol and glucose was found by Ambujom, who observed that a consortium consisting of eight phenol degraders and two non-phenol degraders showed simultaneous degradation of phenol and glucose, and the phenol removal was even enhanced 16-20% (Ambujom, 2001). Unfortunately, the change of the abundance of the single consortium members as result of the additional glucose feeding was not studied. But the diauxic pattern of glucose and phenol degradation indicates that not one of the non-phenol degraders increased in abundance, but that a strain capable of phenol degradation used the glucose as a carbon source.

All these examples show that often consortia are able to cope with the stress of mixed substrate utilisation. When the 4-CS degrading consortium was challenged with mixed substrate utilisation, a secondary degrader metabolised the second carbon source. This reaction can generally not be performed by a single strain, although exceptions exist (Zaidi & Metha, 1995).

4.3.2.2 Response limits: System breakdown

The boundaries of the efficiency of the 4-CS degrading consortium were found when ethanol or iso-propanol were applied as second carbon sources. A system breakdown resulted. The development of the stress upon the consortium can also be found in the description of chemical stress by Ford (1989). The author states that the earliest phase of ecosystem response begins with the loss of species most sensitive to the stressor. After three days of additional ethanol feeding, the abundance of *P.* sp. MT 1 decreased. But not only was a decrease in abundance observed, in addition the strain stopped degrading 4-CS. The consequences of the loss of species depend on the role played by the stressor-sensitive species and the amount of redundancy that exists in this role. As *P.* sp. MT 1 is the only primary degrader, the loss of this species resulted in the stop of 4-CS degradation. Compositional changes due to native species which respond in individualistic ways were observed, and similarly the abundance of *P.* sp. MT 4 increased. Finally, the decline in species richness was accompanied by decrease in biomass and abundance. The functional consequences of these structural changes depend primarily on the structural organisation and trophic dynamics of the receiving system. The additional feeding of ethanol resulted in the inhibition and washout of

the selective feeder of the consortium, *P.* sp. MT 1, and at this point the 4-CS degradation stopped.

Generally, is the result of the influence of a second easy to degrade carbon source the inhibition of the degradation of a pollutant by a pure culture (Goldstein et al., 1985). Exemplary was the degradation of *para*-nitrophenol by *Pseudomonas* strain GR inhibited by the additional feeding of glucose (Zaidi & Metha, 1995). Zaidi concludes that whether a strain might continue to degrade successful pollutants depends on the strains ability to compete for other easily utilisable compounds. If the strain can not compete for the second substrate, the strain continues pollutant degradation. In the continuous culture experiment with ethanol or iso-propanol addition, *P.* sp. MT 1 can not use the alcohols as carbon source (see chapter 3.3.1.1). Nevertheless the 4-CS degradation stopped.

Shimao et al., who observed a consortium structure shift and decreasing degradation of polyvinyl alcohol due to ethanol addition, named competition for a certain nutrient and production of a growth inhibitor as possible reasons (Shimao et al., 1985). No accumulation of a toxic product was observed in the additional ethanol feeding experiment of the 4-CS degrading consortium. Therefore the best explanation for the stop of 4-CS degradation in the ethanol addition experiment is the inhibition of enzymes of *P.* sp. MT 1 by the alcohol.

Other cases in which a consortium was not able to cope with the addition of a second substrate were found in the literature. Suppression of biodegradation of one compound by a second had been previously noted (Harder & Dijkhuizen, 1982; Schmidt & Alexander, 1985). This could be a result of inhibition by the second compound, conversion to a toxic product, deficiency of O_2 or competition for limiting nutrients.
Reasons based on biotic influences were named by Egli (1995). Competition could be one reason; in general, when two strains are competing, the one with the wider range of carbon substrates should outcompete the strain with the smaller substrate range (Egli, 1995; Gottschal, 1983). The network of the carbon flux through the consortium could be another reason. The substrate fluxes through the species could be altered. This could result in the accumulation of toxic products or in a kinetic unfavourable condition of one consortium member. In the alcohol addition experiment no accumulation of toxic metabolites was observed.

In summary:
It is apparent, that the addition of easily utilised carbon sources always had an effect upon the structure of the consortium. An example is the additional feeding of $^1/_{10}$ NB, which resulted in a drastic increase of the consortium member *E. brevis* MT 2. This strain had no defined role in the consortium, but was always about 2% in abundance. This strain became the major $^1/_{10}$ NB

degrader and increased to 50% in abundance, whereas the rest of the consortium continued to degrade 4-CS. It was expected that the additional feeding of an easily utilised carbon source would result in an effect on the function of the consortium. It was expected that the consortium would stop degrading 4-CS and only use the easily utilised carbon source. But simultaneous degradation was observed when histidine and $^{1}/_{10}$ NB (and 4-CC) were fed in addition to 4-CS. The effects of the carbon sources were based on the nature of the carbon source, whether or not they were inhibitory and whether they could use the carbon source to gain energy. But the reactions of the pure strains were not always sufficient to describe the reaction of the consortium after the start of the additional feeding, e.g. the pure culture of *P*. sp. MT 1 degraded histidine fastest, but *P*. sp. MT 4 was the main degrader of histidine additionally fed to the consortium. Therefore the effects of the carbon sources upon the structure and function of the consortium were based on the reactions of the single consortium members, and these affected the interactions of the whole consortium. Other interactions of the consortium members, such as production of toxins, were not observed but can not totally be excluded. It is possible that one consortium member, which degraded the second carbon source, stopped to perform its role in the 4-CS degradation network. This should result in the accumulation of metabolites, but this was not observed.

4.4 Competition of consortium with optimised degraders

Biodegradation of xenobiotics is often biased by the abilities of pure cultures to degrade the pollutant, e.g. because the strain contains only a part of the degradation pathway. One approach is to combine single strains to a mixed culture, as in the case of the here studied 4-CS degrading consortium; the other approach is to expand the catabolic abilities of single strains (by prolonged cultivation with the xenobiotic of interest or by genetic engineering) to enable them to degrade the xenobiotic. Inoculation experiments with such optimised strains often resulted in the failure of biodegradation (Bailey et al., 1999; Goldstein et al., 1985). The continuous culture of 4-CS degrading consortium provided an excellent tool for studying the introduced strains' ability to survive and perform the degradative function, and for studying its influence upon the consortium.

P. sp. MT 1 as only consortium member contains the enzyme salicylate 1-hydroxylase besides a catechol *ortho*-cleavage pathway. *P. putida* A02 and *P. putida* G7::4/4 are also able to activate chlorosalicylates for ring-cleavage, but these strains contain additionally the chlorocatechol *ortho*-cleavage pathway and thus an enzyme equipment supposed to be optimal for chlorocatechol mineralisation. Hence contain the environmental isolate *P. putida* A02 and the genetically engineered strain *P. putida* G7::4/4 a superior enzymatic composition compared to single members of the consortium. In batch culture, they could tolerate higher concentrations of 4-CS, and the degradation rates were higher compared to the main degrader *P*. sp. MT 1 and the whole consortium. Therefore it was expected that the strains would be able to outcompete one of the consortium members or the whole consortium.

But the results of the experiments showed that although both strains were able to grow, *P. putida* A02 was washed out and *P. putida* G7::4/4 became a new consortium member with an abundance of 14%.
These results show that it is not sufficient to observe the kinetic abilities of single strains to predict their ability to be competitive. Obviously, other mechanisms such as the interaction of consortium strains (probably based on the 4-CS degradation network) have a more dominant influence upon the consortium stability. To break up this strong society it is not enough to add a competing strain. The results of the competition experiments showed that the function of the consortium was not negatively influenced by the competition attack of optimised degrader strains.

Comparison with other works is difficult, as either chemostat competition experiments with two competing strains were performed, or the survival of a competitor in activated sludge or soil of unknown community composition was studied. The results can not be directly compared, as soil or activated sludge provide a different environment than the chemostat system. But the reasons for the success or failure of inoculation can be tested to be applicable for the chemostat system as well.

4.4.1 Washout of environmental isolate *P. putida* A02

The environmental isolate *P. putida* A02 was washed out. Other studies resulted in the fact that the inoculant could not prevail in a community.
Goldstein et al. studied the survival of the soil isolate *P.* sp. strain 1 capable of mineralising 2,4-dichlorophenol (DCP) (Goldstein et al., 1985). Addition of the strain to sterile soil resulted in evident degradation of 10 µg DCP per g, but no mineralisation was observed in unsterile soil. The author suggested that microorganisms able to degrade organic pollutants in culture may sometimes fail to function when inoculated into natural environments because the concentration in nature may be too low to support growth, or because the organisms may be susceptible to toxins or predators in the environment, or because the strains may use other organic compounds in preference to the pollutant, or may be unable to move through soil to sites containing the chemical. Most of these arguments can not explain the washout of strain *P. putida* A02, as the concentration of strain and the concentration of the substrate 4-CS should be sufficient in the chemostat system, and no other carbon source or predator or occurrence of toxins was observed. But one possibility is that *P. putida* A02 could not achieve enough substrate for sufficient growth.
Trevors et al. studied the cell numbers and distribution of bacteria released into soil. The authors found, that biotic and abiotic factors are significant in controlling the success of applications of microbial cells in soil (Trevors et al., 1994). As the abiotic factors are controlled in the chemostat system and the strain *P. putida* A02 was adapted to these

conditions, these factors fail to be the reason for the washout. Biotic reasons (for example competition with other strains for substrate) remain.
Pritchard studied the use of inoculation in bioremediation (Prichard, 1992). Interesting aspects, that should be considered when inoculation is attempted, are adaptation, high inoculant concentration, inoculant formulation, the availability of ecological niches and colonisation success. It is necessary to use environmentally competent strains, but even these are going to be restricted in their ability to perform their function due to competition. Two aspects do apply for the washout of *P. putida* A02. The chemostat did not provide a ecological niche for the introduced strain (in contrary to later mentioned bioprotection studies, where the introduced strains have the ability to degrade specific chemicals, which can not be degraded by the indigenous populations), and *P. putida* A02 had to compete with the consortium and its single members. Probably the strain could not grow as fast as in the axenic culture due to the existing network of the consortium, it lost the competition for the substrate. One possible explanation might be that the affinity for 4-CS of *P. putida* A02 was lower compared to *P.* sp. MT 1. Another point is the adaptation, in the experiment displayed in Chapter 3.4.3, *P. putida* A02 was pregrown in batch culture and inoculated into continuous culture, therefore the strain was not as well adapted to continuous culture conditions. But after an adaptation phase the strain did grow, but the observed growth rate was below the growth rate observed in pure culture studies and below the dilution rate.
Ka et al. found that species interactions affected competition in soil and axenic broth. The author studied one constructed strain and three environmental isolates which can degrade 2,4-dichlorophenoxyacetic acid in competition in axenic broth, mixed broth and unsterile soil (Ka et al., 1994). Two of the environmental isolates, which outcompeted the other strains in the axenic broth, could not compete in the soil competition experiment. The authors argued that the rapid exchange of cell products in the mixed broth reduced the fitness difference, but this did not occur in the unmixed physically isolated niches present in the soil environment. This argument does not apply for the washout of strain *P. putida* A02, as the chemostat experiment had a liquid matrix.

In summary the function of the consortium was not affected by the addition of strain *P. putida* A02, because 4-CS degradation continued. Even though the strain grew, the observed growth rate was higher as the theoretical washout rate (which would occur without growth of cells), but below the dilution rate and therefore was *P. putida* A02 washed out. This observation is contrary to the observation of the pure culture, where the growth rate of *P. putida* A02 was higher than that of *P.* sp. MT 1. Possible reasons are that *P. putida* A02 had a lower affinity for 4-CS as *P.* sp. MT 1.

4.4.2 Strains *P. putida* G7::4/4 and *P.* sp. B13 SN45P became new consortium members

The addition of the genetically engineered strains *P. putida* G7::4/4 and *P.* sp. B13 SN45P resulted in the co-existence of both strains under steady state conditions.

Table 16: Consortium composition resulting from addition of strain *P. putida* G7::4/4 and from 4-CS concentration increase to 10 mM and addition of strain *P. putida* G7::4/4

disturbance	**MT 1**	**MT 2**	**MT 3**	**MT 4**	**G7::4/4**
undisturbed (5 mM 4-CS)	76%	2%	19%	3%	
5 mM 4-CS + G7::4/4	63%	2%	19%	2%	14%
10 mM 4-CS	79%	2%	18%	1%	
10 mM 4-CS + G7::4/4	59%	3%	14%	1%	23%

The addition of *P. putida* G7::4/4 to the consortium growing on 5 mM 4-CS in continuous culture resulted in competition of *P. putida* G7::4/4 with *P.* sp. MT 1, because the abundance of *P. putida* G7::4/4 and of *P.* sp. MT 1 sum up to the abundance of *P.* sp. MT 1 without disturbance (see Table 16). The increase of the 4-CS concentration to 10 mM and the addition of *P. putida* G7::4/4 resulted in a consortium composition with lower abundance of *P.* sp. MT 1 and *A. xylosoxidans* MT 3. The reason is probably that less carbon source is available for *A. xylosoxidans* MT 3. Without a competing strain, *P.* sp. MT 1 metabolises 100% 4-CS and 10% from this spills out as 4-CC to be metabolised by *A. xylosoxidans* MT 3. When *P. putida* G7::4/4 competes with *P.* sp. MT 1 for the carbon source, only approximately 80% 4-CS can be metabolised by *P.* sp. MT 1, because the rest is used up by *P. putida* G7::4/4. Therefore only 8% 4-CC can be used by *A. xylosoxidans* MT 3 as carbon source, therefore the abundance of *A. xylosoxidans* MT 3 is also lower.

Table 17: Consortium composition resulting from growth on 5 mM 4-CS and 5 mM 4-CC and from growth on 5 mM 4-CS and 5 mM 4-CC and addition of strain *P.* sp. B13 SN45P

disturbance	**MT 1**	**MT 2**	**MT 3**	**MT 4**	**B13 SN45P**
undisturbed (5 mM 4-CS)	76%	2%	19%	3%	
	4.9×10^{8}	1.1×10^{7}	1.1×10^{8}	2.3×10^{7}	
5 mM 4-CS + 5 mM 4-CC	69%	1%	28%	2%	
	nd	nd	nd	nd	
5 mM 4-CS + 5 mM 4-CC	60%	1%	25%	4%	10%
+ *P.* sp. B13 SN45P	8×10^{8}	1.3×10^{7}	3.7×10^{8}	2.6×10^{7}	8.4×10^{7}

relative abundance expressed as percentage; nd = not determined; absolute abundance expressed as cells ml^{-1};

The addition of 5 mM 4-CC resulted in a consortium composition with higher abundance of *A. xylosoxidans* MT 3 (see Table 17). The absolute abundance of *P.* sp. MT 1 is also higher as it would be if *P.* sp. MT 1 would only metabolise 4-CS. Therefore can both strains use 4-CC as substrate. *P.* sp. MT 1 contains the enzyme catechol 1,2-dioxygenase, this enzyme has a lower affinity for 4-CC compared to the enzyme chlorocatechol 1,2-dioxygenase of *A. xylosoxidans* MT 3. However, the cell concentration of *P.* sp. MT 1 is 4 times higher than that of *A. xylosoxidans* MT 3. Therefore was about half the 4-CC metabolised by *P.* sp. MT 1. The addition of a third competitor, *P.* sp. B13 SN45P, resulted in a lower relative abundance of *P.* sp. MT 1 and *A. xylosoxidans* MT 3.

Which strain is able to compete best for the substrate depends certainly on the substrate affinity of the involved enzymes.

During the period of observation of the experiments, *P. putida* G7::4/4 and *P.* sp. B13 SN45P neither outcompeted one of the consortium members nor the whole consortium, and neither were they washed out. The strain *P. putida* G7::4/4 had a trait genetically introduced, which was necessary for the ability to use 4-CS as a carbon source. *P.* sp. B13 SN45P carried (for the here tested environment) an superfluous genetically introduced trait.

One one hand, one possible result was that the GEMs were washed out. Lenski states that "genetically modified microorganisms should be less fit than their unmodified counterparts" (Lenski, 1993), because of energetic inefficiency, disruption of genetic co-adaptation and domestication. But the experiments of this thesis show that the *P. putida* G7::4/4 and *P.* sp. B13 SN45P were not washed out.
Bailey et al. found that the reasons for variable success of the release of recombinant microorganisms include loss of ecological competence; spontaneous mutation either of the gene itself of global regulatory genes that control the expression of secondary metabolites; variable expression of the trait (introduced or nascent) due to local environmental conditions that impact on cellular metabolism; and poor survival, lack of fitness, and inability to compete (Bailey et al., 1999). In field release studies, biotic and abiotic environmental factors play a major role. In the chemostat system used in this thesis, abiotic factors were controlled and did not vary. None of the other mentioned factors hindered the survival of the introduced GEMs in this thesis.
McClure et al. observed that *P. putida* UWC1, with or without the recombinant plasmid pD10, stabilised in an activated sludge unit after an initial rapid decline at between 10^4 and 10^5 CFU ml^{-1} (McClure et al., 1991). The presence of 3-chlorobenzoate had a detrimental effect on the strain, with or without plasmid. McClure surmised that the occurrence of toxic metabolites might be one reason. *P. putida* G7::4/4 and *P.* sp. B13 SN45P did survive, because no toxification due to metabolites occurred. No peaks did occur in the area of

protoanemonin or similar compounds in the HPLC measurement. In addition, the occurrence of chlorocatechol or catechol would have been monitored by HPLC or would have turned the liquid brown, but this was not observed.

McClure et al. considered that the occurrence of a natural (enriched) 3-CB degrading population has more influence. This resembles the set-up of this thesis, where the natural consortium was able to degrade 4-CS. In McClure's experiment, the introduced strain could not compete with the natural 3-CB degrading population (McClure et al., 1991). The reason for this was that the introduced strain was not well adapted to the environmental conditions. A similar observation was made by Boon et al., who observed survival of a *Comanomonas testosteroni* strain, supplied with an additional green fluorescent protein (GFP)-gene in activated sludge (Boon et al., 2000). The strain grew faster than the washout and predation rate and had the ability to form dense flocs under unfavourable conditions. The strain was active and degraded 3-chloroaniline (3-CA) for three weeks. At this point 50% removal was observed, along with a declining cell density. The authors refuted to the hypothesis that the strains stopped 3-CA degradation due to the availability of alternative substrates, as 3-CA degradation was not repressed by other substrates (LB plus 3-CA). The control reactor showed no decrease in banding pattern, and no drastic changes in the reactor performance were observed due to the addition of 3-CA. Boon et al. stated that the sludge had time to adapt. After the adaptation of the natural community, this community was able to degrade 3-CA. The introduced strain in Boon's work failed to compete with the community. These two examples show conditions similar to the ones used in this thesis, as the natural communities had degradative abilities comparable to the introduced strain. But in contrast to the work of McClure et al. and Boon et al., the strains *P. putida* G7::4/4 and *P.* sp. B13 SN45P were not washed out. Although the competitors *P. putida* G7::4/4 and *P.* sp. B13 SN45P were pre-grown in batch culture, after 10 days they grew in the continuous culture. Therefore, they were able to adapt to the continuous culture conditions.

Watanabe et al. studied the population dynamics of two phenol-degrading bacteria, *P. putida* BH and *Comamonas* sp. strain E6, that had been introduced into phenol-digesting activated sludge (Watanabe et al., 1998). A initial rapid decline was followed by a slower decline. The authors explained the slower decline as incorporation into activated-sludge flocs. Pipke et al. showed survival and function of a genetically engineered *Pseudomonad* in aquatic sediment microcosms (Pipke et al., 1992). The strain successfully colonised the sediments. Incorporation in activated sludge flocs or sediment does not apply to explain the survival of the GEMs in the here discussed experiments, as the consortium was maintained in liquid continuous culture. Nevertheless were the above mentioned strains able to adapt to environmental conditions, which were necessary for the survival of the strains.

The dominating stress in the chemostat system is the availability of the carbon source. *P. putida* G7::4/4 and *P.* sp. B13 SN45P were not washed out because the strains found access to the carbon source (4-CS for *P. putida* G7::4/4 and 4-CC for *P.* sp. B13 SN45P).

On the other hand, it was expected that *P. putida* G7::4/4 would dominate the consortium or outcompete one of the consortium members, as the strain could tolerate higher concentrations of 4-CS and the degradation rates were higher compared to the main degrader *P.* sp. MT 1 and the whole consortium.

Huertas et al. observed that three *P. putida* strains were able to survive in unsterile soil, and the addition of 10% (v/wt) toluene led to an immediate decrease, but a recovery of these strains occurred (Huertas et al., 1998). A interesting observation, which was not further discussed, was that the strain *P. mendocina* KR1, which was the winning strain in competition assays in C-limited chemostats, was not able to become established in nonpolluted soil. This finding shows that competition assays performed in the laboratory cannot always be extrapolated to a complex environment, such as soil. In the here discussed experiment the kinetic parameters for 4-CS degradation could not predict the outcome of competition experiments under chemostat conditions.

After introduction of *P. putida* G7::4/4, the abundance of the strain under steady state conditions was 14%. When the double amount of carbon source was available, the absolute and the relative abundance of this strain increased (see Chapter 3.4.5 and Table 12). Due to stable kinetic parameters, the interaction between the strain and the other consortium members is probably based on the role of the carbon flux of 4-CS. If this is true, the doubling of the carbon source should result in the same relative consortium composition, but in the doubling of the absolute cell number of *P. putida* G7::4/4 (if no toxic metabolites occur). But comparison to the addition experiments of the consortium growing on 5 mM 4-CS (see Table 12), reveals that *P. putida* G7::4/4 displays a higher average relative abundance (expected 14%, found 23%) and a higher average absolute abundance (expected 2×10^8 cells ml^{-1}, found 3.8×10^8 cells ml^{-1}). As in the previous experiment, the change in the consortium structure was mainly based on the lower abundance of the strain *P.* sp. MT 1, but also the abundance of *A. xylosoxidans* MT 3 was reduced from 19% to 14%.
P. putida G7::4/4 did not dominate the consortium and did not outcompete one of the consortium members because the strain had not the ability to get access to the whole carbon source. The main part of the 4-CS was still metabolised by the consortium.
The occurrence of the same or different pathways for the degradation of one substrate provides stability for a consortium. Chapin et al. concluded that the abundance of species with similar ecological effects should give stability to ecosystems (Chapin III et al., 1997). The results of the study of Hashsham et al. indicated that stability is linked to community flexibility as reflected in the ability to shift the electron and carbon flow through various alternative guilds in an efficient manner (Hashsham et al., 2000). The incorporation of *P. putida* G7::4/4 and *P.* sp. B13 SN45P would provide such a redundancy of pathways and probably have a stabilising effect upon the consortium.

Some studies address the question of bioprotection. This involves the introduction of a genetically engineered microorganism, which had a specific trait introduced, which enabled the strain to degrade a specific substrate which often harmed or influenced the naturally occurring microorganisms. In bioprotection studies often the soil community or the activated sludge community lacked this trait. One example for such successful bioprotection experiments is the bioprotection of activated sludge from pollutant shocks (Eichner et al., 1999; Erb et al., 1997). The authors observed that the microbiota of the non inoculated control reactor collapsed after the shock load of xenobiotics, with results indicating loss of species. The GEM survived and protected the community. But in these cases, an ecological niche for the introduced strain existed, and therefore the strain didn't have to compete with the natural microflora. The competitor addition experiments studied in this thesis differ from these experiments. In the experiment shown here, the consortium was able to degrade 4-CS and the introduced GEM had a small advantage (*P. putida* G7::4/4 contains better enzyme composition than *P.* sp. MT 1, but similar enzyme composition as the whole consortium) or the same genetic composition (*P.* sp. B13 SN45P can be compared to *A. xylosoxidans* MT 3). Therefore the prerequisites were different.

4.4.3 GEMs were more competitive than environmental isolate

The genetically engineered microorganisms were able to survive better in the consortium than the environmental isolate *P. putida* A02. It made no difference upon the survival, whether the trait, which was genetically introduced, was necessary for the ability to use 4-CS as a carbon source (*P. putida* G7::4/4), or whether the genetically introduced trait (for the here tested environment) was superfluous (*P.* sp. B13 SN45P).

Halden et al. studied three bacteria that degrade 3-phenoxybenzoic acid (3-POB), which were added to soil microcosms containing 3-POB (Halden et al., 1999). The environmental isolate, *P. pseudoalcaligenes* POB310, remained detectable in soil that contained 50 ppm of 3-POB, only the 3-POB degradation was incomplete. The two modified *Pseudomonas* strains, B13-C5 and B13-ST1, derived from strain B13, not only survived, but the density increased and 3-POB was totally degraded. The conditions in the here discussed experiment for *P. putida* A02 and *P. putida* G7::4/4 were more difficult. In Halden's experiment, the soil microflora was only able to degrade 3-POB minimally, whereas *P. putida* A02 and *P. putida* G7::4/4 had to compete with the consortium for 4-CS. Still, in Halden's experiment the genetically engineered organism performed better than the environmental isolate as was also apparent in this study. The strain B13 was shown to be fairly robust and to survive in a variety of habitats (Nusslein et al., 1992; Pipke et al., 1992). The strain POB310 survived poorly in soils. In addition strain B13-D5 exhibited a slightly greater affinity (lower K_S) for 3-POB and had a better growth yield. In Halden's experiment the kinetic parameters mirrored the ability to compete, and the strains with better parameters were able to compete better. The important

feature is probably the greater affinity for the substrate, which was supposed previously (Chapter 4.4.1) as one criterion influencing the ability of an introduced strain to compete.

4.4.4 Effects of GEMs on microbial populations

Doyle et al. studied the effects of genetically engineered microorganisms on microbial populations and processes in natural habitats (Doyle et al., 1995). The authors found, that the introduction of GEMs has the potential to affect the structure and function of ecosystems. GEMs have been shown to (1) compete successfully with the indigenous microbiota of disturbed ecosystems; (2) transfer their novel genes *in situ* with the expression of these genes in a new host; (3) affect metabolic activities and the rate of biomass turnover; (4) influence the community structure and function of the indigenous microbiota in various habitats; (5) affect interactions between symbiotes and organisms at different trophic levels; and (6) produce metabolites that may have unanticipated impacts on the environment. The influences of GEMs upon the 4-CS degrading consortium, which were studied in this thesis, led to changes in the community structure, as was also observed by Doyle (Point 4). The author also determined the critical point, which defined whether the competition of the GEM was successful. For a GEM to affect the stability or expression of a population, it must disrupt existing stabilising factors. These factors are either abiotic or biotic, or they arise from the dynamics of "meta-populations" (group of indigenous populations). Often these factors are unknown, making it difficult to predict whether or not a GEM is able to compete. The stabilising (consortium structure defining) factor of the 4-CS degrading consortium was the 4-CS degradation network. Whether or not the GEM was able to gain access to the carbon source (or to metabolites) should determine, whether the GEM was able to survive.

The competition experiments show that the consortium can not be easily challenged by the introduction of a competitor. The consortium was very stable, as it continued to degrade 4-CS. The environmental isolate *P. putida* A02 was washed out, and two GEMs became new consortium members. Although competition occurred, as the abundance of *P.* sp. MT 1 was lower after addition of *P. putida* G7::4/4 and the abundance of *P.* sp. MT 1 and *A. xylosoxidans* MT 3 was lower after the addition of *P.* sp. B13 SN45P, this did not lead to the extinction of any consortium members or of the GEM. The mechanism on which the reaction is based is unknown. Probably due to carbon flux through the consortium network, the competitors were not able to completely take away the substrate. It is possible that *P.* sp. MT 1 has the highest affinity for 4-CS. *P. putida* G7::4/4 and *P.* sp. B13 SN45P were incorporated in the consortium structure. It is highly probable that the new consortium member has a newly defined role in the carbon flux of the consortium. The consortium's ability to coexist with the invader is probably to the benefit of all members, as metabolic redundancy is achieved, which in all likelihood enhances the stability of the system.

4.5 Reaction to disturbance by reduced oxygen concentration

As a value to describe the efficiency of the chemostat to transport oxygen into the reaction liquid, was the k_La value estimated by the dynamic method. The k_La value of the chemostat was 2.13 to 2.16 × 10^{-3} s^{-1} (see 2.5.5.6). Compared to other values in the literature, the k_La value of the chemostat was very small. The k_La is described in the literature as being in the range of 0.02 to 0.25 s^{-1} (Doran, 1998). The value of the chemostat employed in this thesis, aerated by the air influx of the bottom of the fermenter, is 2.1 × 10^{-3} s^{-1}, which is one magnitude lower. Nevertheless, the air influx had to be reduced massively (to 0.006 vvm) to reach limiting areas. Therefore the air influx under undisturbed conditions is still sufficient, as the consortium cells have a relatively low growth rate and consume substrate and oxygen slowly, and the chemostat is substrate limited.

The oxygen concentration in the disturbed chemostat was reduced to 80%, 60%, and to 10% oxygen saturation. Twice even lower oxygen saturation was achieved by oxygen decrease due to cell growth, once 8% and once 2%. The concentration of oxygen at 100% saturation in water at 1 bar and 12°C is 10.8 mg O_2 l^{-1} (Greenberg, 1965); therefore is the oxygen concentration at 10% saturation 0.11 mg O_2 l^{-1}. The critical oxygen concentration, which is needed to induce aerobic hydrocarbon concentration, depends on the type of hydrocarbon(s) and the specific microbial population, and was reported to be in the range of 1.0 - 1.5 mg O_2 l^{-1} (Wilson & Bouwer, 1997) and 0.05 to 1 mg l^{-1} (Yerushalmi et al., 2001). Therefore was a limiting range reached by the oxygen reduction experiment.
The lowest oxygen concentration values which were reached in the oxygen limitation experiment were 2% and 5% saturation. This corresponds to an oxygen concentration of 6.8 µM O_2 l^{-1} and 17.1 µM O_2 l^{-1}, respectively. The Michaelis constants (K_m) of catechol- and chlorocatechol 1,2-dioxygenases from *P.* sp. B13 for O_2 with 4-chlorocatechol were found to be 400 and 486 µM, respectively (Dorn & Knackmuss, 1978b). Therefore the oxygen concentration reduction to 2% and 5% was oxygen stress for the consortium.

The reduction of oxygen concentration to below 10% saturation had no influence upon the consortium structure. 4-CS was continuously degraded. However, the reduction of the oxygen concentration resulted in the occurrence of a yellow colour in the chemostat (Chapter 3.5). This is due to accumulation of 5-chloro-2-hydroxymuconic semialdehyde, a *meta*-cleavage product. A test of the enzyme activity of the raw extract of the disturbed and undisturbed chemostat indicated higher *meta*-cleavage activity in the disturbed chemostat (Chapter 3.5.1). The production of a *meta*-cleavage product with 3-chlorocatechol as substrate was previously observed. Riegert et al.found that 3-chlorocatechol was transformed to a yellow product, which was identified as 3-chloro-2-hydroxymuconic semialdehyde (Riegert et al., 1998). Riegert et al. found that the absorption spectrum changed from 378 nm at neutral to 335 nm at low pH. The yellow product observed under oxygen limitation in this thesis is most likely

5-chloro-2-hydroxymuconic semialdehyde, the *meta*-cleavage product of 4-chlorocatechol. Production of 5-chloro-2-hydroxymuconic semialdehyde from 4-chlorocatechol has been previously observed (Farrell & Quilty, 1999; Wieser et al., 1994).
Meta-cleavage activity with catechol as substrate was below 1%, both in the undisturbed and the disturbed chemostat (see Table 13). Pelz did not observe *meta*-cleavage activity with catechol as substrate in his study of the same consortium under undisturbed conditions (Pelz, 1999a). *Meta*-cleavage activity with 4-CC as substrate could be observed in the disturbed chemostat (see Chapter 3.5.1). It could not be observed, whether *meta*-cleavage activity did or did not occur in the undisturbed chemostat. To differentiate between *meta*- and *ortho*-cleavage enzymes, the *ortho*-cleavage enzymes were inactivated by boiling at 50°C (see Materials and Methods 2.7.3). This treatment might have also harmed the *meta*-cleavage enzymes, which would result in the observation of no activity. But if activity is measured, this is clear evidence for the enzymes being active. Therefore, in the chemostat with reduced oxygen, the *meta*-cleavage enzymes were active. The use of anti-metabolites to inactivate the enzymes is not possible because both enzymes are able to use the same substrates.

The consortium reacted to the oxygen concentration reduction stress by the activation of a different, additional pathway, the *meta*-cleavage pathway. Under aerobic conditions, monooxygenases or dioxygenases catalyse aromatic compounds by introducing hydroxyl groups into the ring structure (Krooneman et al., 1996; Olsen et al., 1997). The occurrence of a *meta*-cleavage product as a result of oxygen stress was previously described in the literature. Kapley et al. studied the influence of different dissolved oxygen (DO) levels on the utilisation of phenol by *P.* CF600 in continuous culture (Kapley et al., 2001). The authors observed that the accumulation of 2-hydroxymuconate semialdehyde depended on the DO level, with the highest amount of 4 µM at 2 ppm, and the lowest of 0.4 µM at 3 ppm. The suppression of the activity of dioxygenases under microaerophilic conditions was previously observed. Krooneman et al. showed that catechol 1,2-dioxygenases (*ortho*-cleavage), under aerobic conditions degrading 3-chlorobenzoate, were suppressed under reduced O_2 partial pressures (Krooneman et al., 1996). The strain *Alcaligenes* sp. L6, isolated under reduced O_2 partial pressure, possessed the catechol 2,3-dioxygenase (*meta*-cleavage) (Krooneman et al., 1996). Hack et al. showed that low DO concentrations reduced the activity of toluene dioxygenase in *P. putida* (Hack et al., 1994). Villiesid and Lilly reported that the induction of catechol 1,2-dioxygenase (*ortho*-cleavage) of *P. putida* was strongly reduced at lower partial pressures of oxygen (Villiesid & Lilly, 1992). Duetz et al. studied the influence of oxygen limitation on the repression of the TOL pathway and *meta*-cleavage (Duetz et al., 1996). The *meta*-cleavage pathway was found to be susceptible to catabolite repression, and less repression was found under conditions of oxygen limitation between 20% to 35%. These examples support the theory that an alternative or additional *meta*-cleavage pathway is switched on by the consortium as a reaction to the reduced oxygen concentration.

Farrell and Quilty observed that degradation of 4-chlorophenol by a mixed culture led to accumulation of 5-chloro-2-hydroxymuconic semialdehyde, the *meta*-cleavage product of 4-chorocatechol (Farrell & Quilty, 1999). The authors found, that *meta*-cleavage is known to generally result in incomplete metabolism due to the production of dead-end or suicide-metabolites (Schmidt et al., 1983; Reineke et al., 1982; Westmeier & Rehm, 1987). However, recent reports have shown that further metabolism of 5-chloro-2-hydroxymuconic semialdehyde may occur (Hollender et al., 1997; Sung Bae et al., 1996). Farrell and Quilty discussed the further degradation of this substance (Farrell & Quilty, 1999). For example, Mars observed a special *meta*-cleavage enzyme which can degrade chlorobenzene via the *meta*-cleavage pathway (Mars et al., 1997). It may be that in the oxygen reduction experiment of this thesis, the 5-chloro-2-hydroxymuconic semialdehyde was also further degraded or that, due to an increasing oxygen concentration, the metabolite was no longer produced, and the remaining metabolite was washed out.

A possible explanation for the reaction of the consortium could be that the production of 5-chloro-2-hydroxymuconic semialdehyde was a way for the consortium to get rid of the accumulation of more toxic metabolites such as 4-CC, protoanemonin or the substrate 4-CS itself.

4.6 Methodological aspects

Different culture systems, batch, fed-batch and continuous culture, were used to study the consortium. The results of the batch and the fed-batch experiments allowed to predict the outcome of the continuous culture experiments. Although batch and fed-batch studies only reveal a limited amount of information, they are a valuable screening and pre-experimental tool. Nevertheless, the suitability of the batch culture as a screening tool was not satisfactory. The consortium structure of the control differed slightly from the consortium composition of the undisturbed continuous culture experiments, for example in the substrate addition batch experiments (Chapter 3.3.1.2), the abundance of consortium member *P.* sp. MT 4 in the control experiment was 13%, this is high compared to the results of the batch experiments with 1 mM 4-CS (7%), see Chapter 3.2.2. This observation is in all likelihood based on the ability of *P.* sp. MT 4 to grow on cell-debris (Chapter 3.2.3). In the literature, fed-batch experiments among other applications were used to confirm degradative abilities of strains (Balfanz & Rehm, 1991) or communities (studied previously in batch culture) or to enhance the productivity of a system (Collins & Daugulis, 1997). In any case is it necessary to confirm the batch experiment results in continuous culture, because the results may differ (Filonov et al., 1997).

The main advantage of fed-batch and continuous culture is the continuous influence of the selective pressure. The continuous culture provided the most results. Still more information

would be needed to explain the reasons for each reaction of the consortium members. Here some methodological improvements are purposed for future experiments:

In pure culture studies of the consortium members it should not only be studied if a strain can use a carbon source as substrate, but in addition, if this substrate has a negative (inhibitory) influence upon the strains, which can not use it as carbon source. For example it could be studied if the growth of *P.* sp. MT 1 on 4-CS is inhibited by the addition of ethanol. It should be studied, whether toxic or enhancing metabolites are produced by the primary degrader. This could be done in an approach shown by Rogers et al., growing the other strains on filtered medium of the first strain, which is growing with the carbon source of interest (Rogers et al., 2000). The batch studies of the pure cultures are often not sufficient, it would be better to grow the single strains in continuous culture and to study degradation, inhibition and the influence of the second carbon sources in experiments in which the second carbon source is fed in a low rate and signs of inhibition or accumulation of metabolites can be studied.

The set-up of the chemostat was sufficient to produce the results of interest. An improvement would be to measure the gas phase, then the metabolic activity could be measured and calculated, another useful measurement would be to measure the chlorine release. The oxygen influx was found to be sufficient (Chapter 4.5), nevertheless could be increased by application of a better stirring system, for example impellers and a higher stirrer rate. In this thesis the previous fermenter set-up (from Frech's and Pelz's studies) was not changed, as the impact of the changes on the composition and structure of the consortium was unknown. The more important reason was that continuous culture under these, maybe not optimal, conditions meets the criteria named by Brock to ensure good consortium studies. These conditions are well-defined cultures, low nutrient concentrations and slow growth rates (Brock, 1987).

It is necessary to monitor not only the carbon source and the second carbon source (it would be better to measure the second carbon source specifically, not only as a sum parameter), but also to monitor always the occurrence of metabolites, especially the first in the pathways, 4-CC, muconate, *cis*-dienelactone and protoanemonin. Pelz has shown that the use of stable isotopes is a good method to study the carbon flux in consortia (Pelz, 1999a). For further experiments it would be good to study not only equicarbonatom amounts of the carbon sources, but also double or triple or half the amount to observe the increasing effects upon the consortium structure.

Competition experiments could be improved by ensuring that the competing strain and the consortium have the same level of fitness. This could be achieved by the parallel maintenance of two chemostats, one inoculated with the consortium and the other with the competitor. Half of the reaction liquid would be exchanged. This would have many advantages: Both, consortium and competitor would be adapted to the growth in continuous culture. It could be observed whether differences would occur, when the competitor is added to the consortium or when the consortium is added to the competitor.

All this work is very time consuming and would extend by far the magnitude of this thesis. The main emphasis of this thesis was to gain first insight in the influences of different disturbances and to provide the possibility to select single influences and reactions for detailed future studies.

4.7 Conclusion

Prior to the study of the influence of changes upon the 4-CS degrading consortium, the consortium under undisturbed conditions was characterised. Caldwell et al. proposes that to confirm whether a community culture has been obtained, it is important to demonstrate that the association of organisms meets criteria of autopoiesis, synergy, communality and homeostasis (Caldwell et al., 1997). Most aspects do apply for the 4-CS degrading consortium. Autopoiesis was shown by the re-association of the consortium from pure cultures of the single consortium member strains and by the development of the expected consortium composition under selective pressure. Synergy could not be proven, as no better kinetic growth parameters of the 4-CS degrading consortium, compared to the pure culture of the primary degrader *P.* sp. MT 1, were observed. One exception was the degradation rates, which were found to be higher for the consortium. But experiments performed in continuous culture with rising dilution rates performed by Pelz (1999a) revealed that the consortium was able to tolerate higher dilution rates than strain *P.* sp. MT 1 alone. Homeostasis implies that the community creates a favourable and stable microenvironment within an unfavourable macroenvironment. Optimised degraders, which were introduced into the consortium were neither able to outcompete the consortium nor one of its members, probably due to this ability of the consortium to create such a favourable microenvironment. The primary test of communality is to determine the response of the community to various environmental gradients. This was the aim of this study to determine, which factors have an influence, and to study the reactions of the consortium to these factors.

It was found that the consortium was stable, as consortium structure and function remained relatively constantly under undisturbed conditions. A similar stable consortium was observed by Ambujom, who studied the composition and stability of a many membered bacterial consortium degrading phenol. The author observed increased phenol degradation under various concentrations and dual substrate feeding by the consortium, which might be due to diverse physiological and biochemical attributes from the combination of more members. Stability of the system was maintained by combined metabolic processes of individual members of the consortium, enhanced by its biomass production, specific growth rate and lack of accumulation of degradation intermediates (Ambujom, 2001). Ambujom's work and the results of this thesis support the view that synergistic and commensalistic relationships between the constituent members accomplished degradative performance of a consortium.

The 4-CS degrading consortium was challenged by the change of environmental factors. All studied factors (second substrate addition, addition of a competitor and oxygen reduction) resulted in an influence upon the consortium. Except for the oxygen reduction experiment and the addition of an environmental isolate, the studied factors always had an influence upon the structure of the consortium. The structure change resulted in some cases in an influence upon the function of the consortium, as in this case the consortium ceased to degrade 4-CS.

The results of the different experiments were:
The addition of a second carbon source always resulted in change of the consortium structure. Addition of $^{1}/_{10}$ NB, 4-CC and histidine resulted in continuous 4-CS degradation. Addition of ethanol or iso-propanol affected the function of the consortium, as the 4-CS degradation stopped.
The addition of competing strains had no influence upon the function of the consortium. The addition of environmental isolate *P. putida* A02 resulted in the washout of this strain. The addition of genetically engineered strains *P.* sp. B13 SN45P and *P. putida* G7::4/4 resulted in a co-existence of the strains. These strains could not dominate the consortium or replace or outcompete one of the consortium members, and neither were the strains washed out.
The oxygen reduction to below 10% saturation had no effect upon the structure of the consortium. The function, continuous 4-CS degradation, was also not affected. But a different pathway to cleave the metabolite 4-CC was switched on. Under the influence of reduced oxygen concentration, *meta*-cleavage activity was observed.

Final explanations for results from the experiments were not always possible. Information about the metabolic network of the consortium allows indication of a missing role of one consortium member if a specific metabolite would accumulate and the abundance of the strain would decrease. For example, if 4-CC had accumulated and *A. xylosoxidans* MT 3 had decreased in abundance, this would have indicated that *A. xylosoxidans* MT 3 was no longer able to perform its role in the consortium network, but such dependencies were not observed in this work, for several reasons: (1) in some experiments, no accumulation of (toxic) metabolites was observed (addition of histidine, ethanol, iso-propanol). (2) In other experiments, measurement by HPLC was hindered (addition of $^{1}/_{10}$ NB). (3) In addition, the sampling period was probably too long, as, for example, accumulated catechol condensated and could not be observed after 24 h (addition of 4-CC). The advantage of this study was to be able to observe the influence of three factors (mixed substrate utilisation, competition and oxygen reduction). Different instrumental set-up (higher biomass, shorter sampling time) would provide the ability to study questions of interest in detail. The continuous culture conditions selected for this thesis were close to the environment as demanded by Brock (1987).

This work helped to identify some of the factors which are necessary to be studied in order to enable development of effective bioremediation processes in the field. The factors which were found to influence the consortium are nutrient supply (organic), oxygen concentration, and microbial competition. Further factors that would be interesting to study are temperature, toxins and other bioactive compounds (organics, heavy metal, surfactants), nutrient supply (inorganic), pH and microbial and macrofaunal grazing. The influence and the limitations due to these factors in specific bioremediation processes have to be studied from case to case, as consortium or community member and pathway composition differs strongly.

Nearly all factors had an influence upon the structure of the 4-CS degrading consortium. Similar observations of the influence of disturbances were previously made. Schimel summarised previous studies of ecosystem consequences of microbial diversity and community structure (Schimel, 1995). The author states that chemical and physical stresses (e.g. pollution) can reduce microbial numbers and diversity to such an extent that the size of specific populations, rather than substrate availability, may become the limiting factor in a range of processes. (In this thesis observed by the system breakdown due to additional feeding of ethanol or iso-propanol). Diversity indices often decrease in response to environmental perturbations. Sometimes the decrease in diversity reflects toxicity and is a measure of the survival of tolerant species; in other cases, the decline in species diversity reflects the success of a limited number of physiologically specialised microorganisms (Atlas, 1984).

Some of these structural changes resulted in effects upon the function of the consortium. Whether or not the change of the consortium structure would result in an influence upon the function of the 4-CS degrading consortium depended on the nature of the disturbance and on the role of the influenced consortium member. A similar conclusion was made by Chapin et al., who stated that the apparent conflict between the perspectives that each species is important, and that there is ecological redundancy among species, is resolved when biotic composition is considered in terms of functional types of organisms and their environmental responses. Changes in the abundance of species that differ in ecosystem consequences should affect process rates or patterns, whereas the abundance of species with similar ecological effects should give stability to ecosystems (Chapin III et al., 1997). The mixed substrate addition experiments confirm this theory. When a secondary degrader (and there were three in the consortium) changed to a primary degrader of the added substrate (for example *P*. sp. MT 4 in the histidine addition experiment), this had no influence on the function of the consortium. But when the primary degrader *P*. sp. MT 1 was affected, for example by ethanol addition, no other strain could replace *P*. sp. MT 1, and the function of the consortium could not be performed further.

The influence of factors upon consortium structure and function was previously studied. The relationship between functional stability and community structure was studied by Fernandez et al. (2000). Methanogenic bioreactor communities were used as model ecosystems, and the effect of a substrate loading shock was studied. He studied two sets of replicated communities, the high-spirochete (HS) set, characterised by good replicability and the low-spirochete (LS) set, characterised by incomplete replicability. In HS communities, glucose perturbation caused a dramatic shift in the relative abundance of community populations, followed by a return to the pre-perturbation community structure. The LS communities were less perturbed. The more stable LS communities were less functionally stable than the HS communities (Hashsham et al., 2000). The authors concluded that in their study, functional stability could not be attributed to higher species diversity or community stability. In general, a higher species diversity should result in a higher flexibility (see below (Morales et al., 1996)). The results indicate that stability is linked to community flexibility reflected in the ability to shift the electron and carbon flow through various alternative guilds in an efficient manner. The 4-CS degrading consortium showed a similar response to the disturbance of oxygen concentration. Under oxygen limitation, the 4-CS degrading consortium was able to employ the *meta*-cleavage pathway, which probably protected the consortium from accumulation of more toxic metabolites.
Franklin et al. stated that ecological diversity, the variety and abundance of species in different habitats and communities, is one of the central themes of ecology (Franklin et al., 2001). Diversity is commonly thought to be a useful indicator of the well-being of an ecological system; however, there is considerable debate over the role diversity plays in ecosystem function (Chapin III et al., 1997; Naeem et al., 1994). Morales et al. and Garland and colleagues used dilution to manipulate microbial diversity for several applications. The various dilution-diversity communities responded differently to invasion attempts (Morales et al., 1996) and to environmental stress (Garland & Lehmann, 1999), with more diverse (less dilute) communities being more stable and better able to withstand invasion. Garland and colleagues studied batch cultures of sterile sewage inoculated with serial dilutions of a sewage microbial community. The autors observed differences in community structure, community size, composition and metabolic redundancy, suggesting that the differences in structure and diversity of communities maintained in the same environment can be manifested as differences in community organisation and function.

As the consortium should serve as a model for an ecosystem, the question arises, whether the generated results can be extrapolated to natural ecosystems. Laboratory experiments and field observations provide different sorts of information. Laboratory experiments are cost effective for studying the mechanisms of individual processes and effects on particular organisms, but they are hard to extrapolate directly to a complex, natural ecosystem. Field observations provide invaluable direct studies of nature, yet they are costly, impossible to control and

difficult to interpret (Gearing, 1989). Although microcosms have been shown to be more comparable to nature in complexity (Wagner-Döbler et al., 1992) and the results are more directly applicable to nature, the possibility of direct application always has to be studied.

The results of this work support the view that consortia (or communities) are feasible for bioremediation processes. Not only does the biodiversity and variety of pathways allow the degradation of compounds which resist the degradation of pure cultures, but these features also provide the basis for stability. But the most valuable ability of consortia (and communities) is that they can react to disturbances and changing of environmental factors, and they posses more mechanisms than pure cultures. One example is the role-switch of a secondary degrader to a second primary degrader which utilises second carbon sources. This reaction could (without few exceptions) not be performed by a pure culture.

5 Abstract

A model microbial community was developed to elucidate environmental parameters which regulate biodegradative activities in pollutant sites. The identification of influential parameters and the study of response mechanisms will support the application and enhance the usefulness of microbial consortia in bioremediation and other related fields. The model was a stable chemostat consortium, which consisted of four bacterial strains, maintained under carbon limited conditions with 4-chlorosalicylate (4-CS) as sole source of carbon and energy. The influence of perturbations (additional carbon sources, changing physicochemical parameters, namely oxygen concentration reduction, and competing bacterial strains) on the structure and degradative function of the consortium was studied. In addition, characteristic kinetic parameters of the consortium were assessed and compared to the very parameters of the main degrader, *P.* sp. MT 1. The chemostat was monitored in terms of the composition of the consortium (by indirect immunofluorescence), the 4-CS concentration, and accumulation of metabolic intermediates of 4-CS degradation, such as 4-chlorocatechol and the antibiotically active protoanemonin (by HPLC).

No significant differences were found comparing the kinetic parameters of the consortium and *P.* sp. MT 1. Probably other parameters explain the consortium cohesion such as the mineralisation of metabolites (which would otherwise become toxic for the consortium) by consortium members. The addition of easily degraded carbon sources, such as histidine or nutrient broth, resulted in a shift of the consortium structure. *P.* sp. MT 4 and , *E. brevis* MT 2 respectively, reached 50% in abundance, and 4-CS was simultaneously degraded. The additional feeding of ethanol resulted in a system breakdown, probably due to the inhibition of primary degrader *P*. sp. MT 1. Here the boundaries of the efficiency of the 4-CS degrading consortium were found. The reduction of oxygen concentration resulted in the activation of a different, additional pathway, the *meta*-cleavage pathway. 4-CS was continuously degraded, but a yellow colour indicated the occurrence of 5-chloro-2-muconic semialdehyde, the product of *meta*-cleavage. The introduction of the engineered strain *P. putida* G7::4/4 or *P*. sp. B13 SN45P, potential competitors with strains of the consortium, resulted in the co-existence of the competing strain and the consortium. An introduced environmental isolate, *P. putida* A02, was washed out.

Almost all factors (except oxygen reduction and addition of *P. putida* A02) influenced the structure of the consortium. The structure change resulted in some cases in an influence upon the function of the consortium (stop of 4-CS degradation).

The ability of one factor to influence the degradation of the consortium was based on the ecological role of the influenced consortium member strain. Changes in abundance of *P*. sp. MT 1, which was the single strain able to perform the first step of the 4-CS degradation, affected the 4-CS degradation. Changes in the abundance of species with similar ecological

effects (like the metabolism of secondary substrates) gave stability to the system, and the 4-CS degradation continued. In addition, stability was found to be linked to community flexibility, reflected in the ability to shift the carbon flow through various alternative pathways. The results of this work support the view that consortia (or communities) are feasible for bioremediation processes, as they posses various mechanisms which enable them to react to environmental changes.

6 Appendix

6.1 List of symbols

6.1.1 List of Symbols

$\triangle S$	Mass of substrate (mg)
$\triangle x$	Mass of cells (mg)
C_{AL}	Concentration of component A in liquid (mg l^{-1})
$\acute{C}_{AL}$	Steady-state concentration of component A in liquid (mg l^{-1})
e	Base of natural logarithms
K_i	Inhibition constant (mg l^{-1})
$k_L a$	Mass-transfer coefficient (s^{-1})
K_m	Michaelis constant (mg l^{-1})
K_S	Substrate constant (mg l^{-1})
n	Number of ...
R	Regression coefficient
r_S	Degradation rate (mol l^{-1} h^{-1} (g dw)$^{-1}$)
S	Mass of substrate (mg)
S_0	Mass of substrate at time 0
t	Time
t_d	Doubling time (h)
x	Cell concentration (mg l^{-1} or cells l^{-1})
x_0	Cell concentration at time 0
Y_{XS}	True yield of biomass from substrate (-)
$Y_{XS,max}$	Maximum yield of biomass from substrate (-)

6.1.2 Greek symbols

τ	Average residence time (h)
γ_P	Degree of reduction of product (-)
γ_S	Degree of reduction of substrate (-)
μ	Specific growth rate (h^{-1})
μ_{max}	Maximum specific growth rate (h^{-1})

6.1.3 List of Abbreviations

C	Carbon
cons.	Consortium
D	Dilution rate
d	Day
degr.	degradation
dw	Dry weight
h	Hour
Km^R	Kanamycin resistance
mM	Milli mole per litre
mo	Microorganism
nd	Value not assessed
NB	Nutrient Broth
P.	*Pseudomonas*
st.	steady state
U	Unit
wa.	washout
wt	Weight

6.2 Mixed substrate utilisation

6.2.1 Additional isopropanol feeding fed-batch experiment

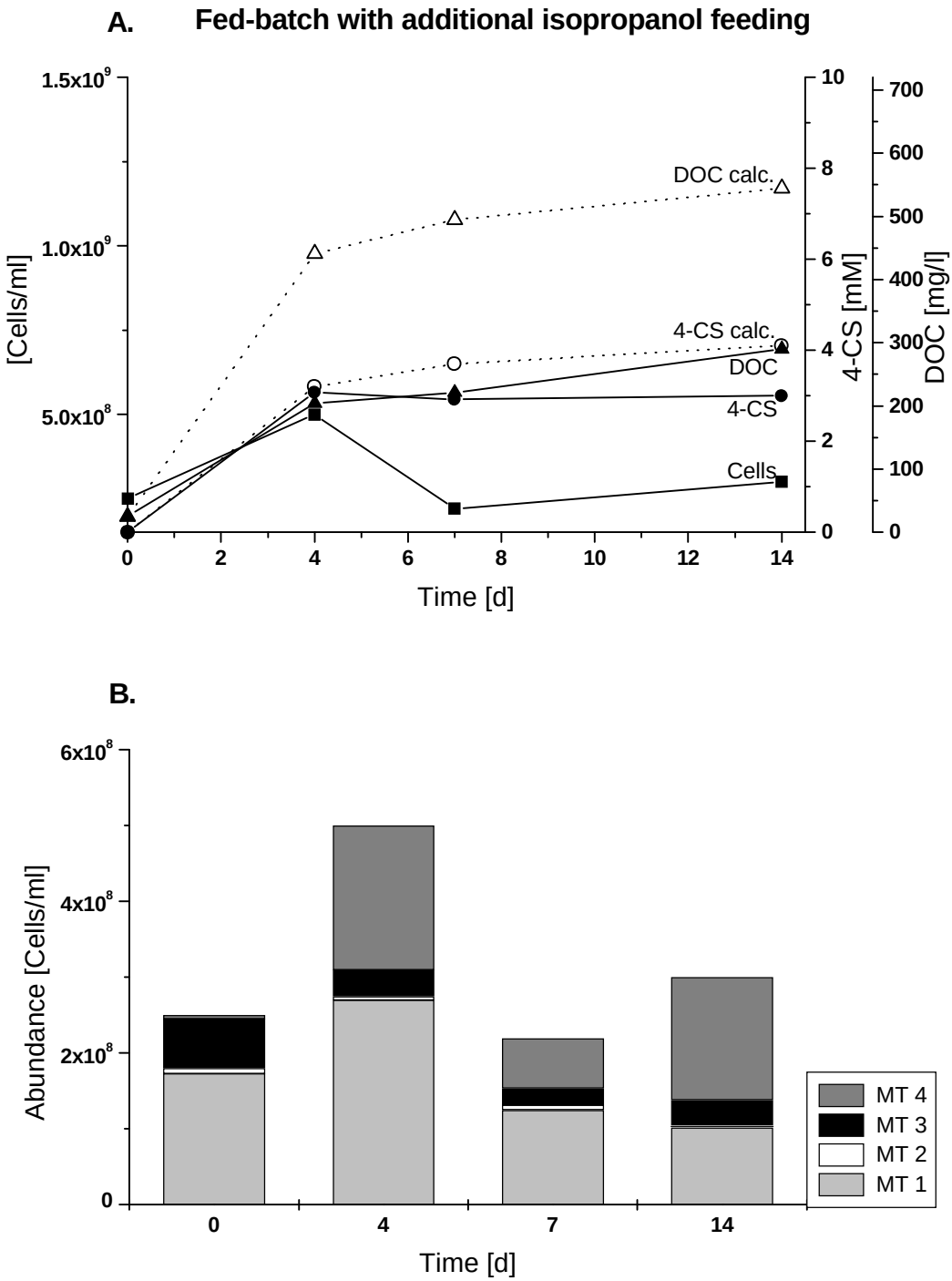

Figure 55: Fed-batch of consortium with 5 mM 4-CS and 10 mM iso-propanol, A. Cell number (■), 4-CS calculated, without degradation (○) and measured (●), DOC calculated, without degradation (△) and measured (▲); B. Absolute abundance of consortium members.

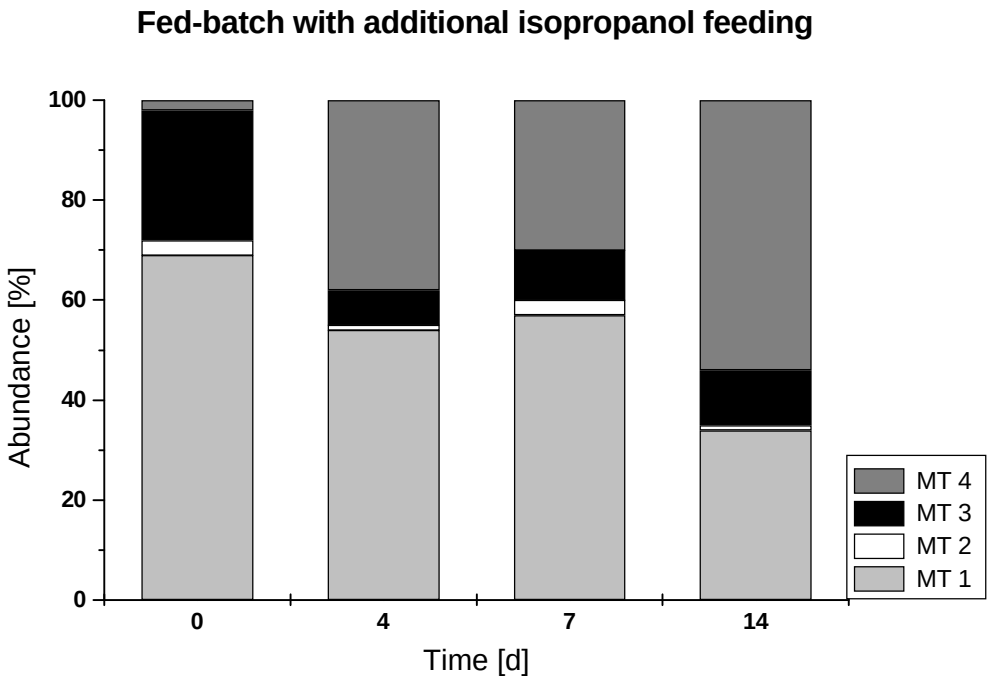

Figure 56: Fed-batch culture of 4-CS degrading consortium with 5 mM 4-CS and 10 mM isopropanol feedstock concentration; Relative abundance of consortium members.

6.2.2 Additional isopropanol feeding continuous culture experiment

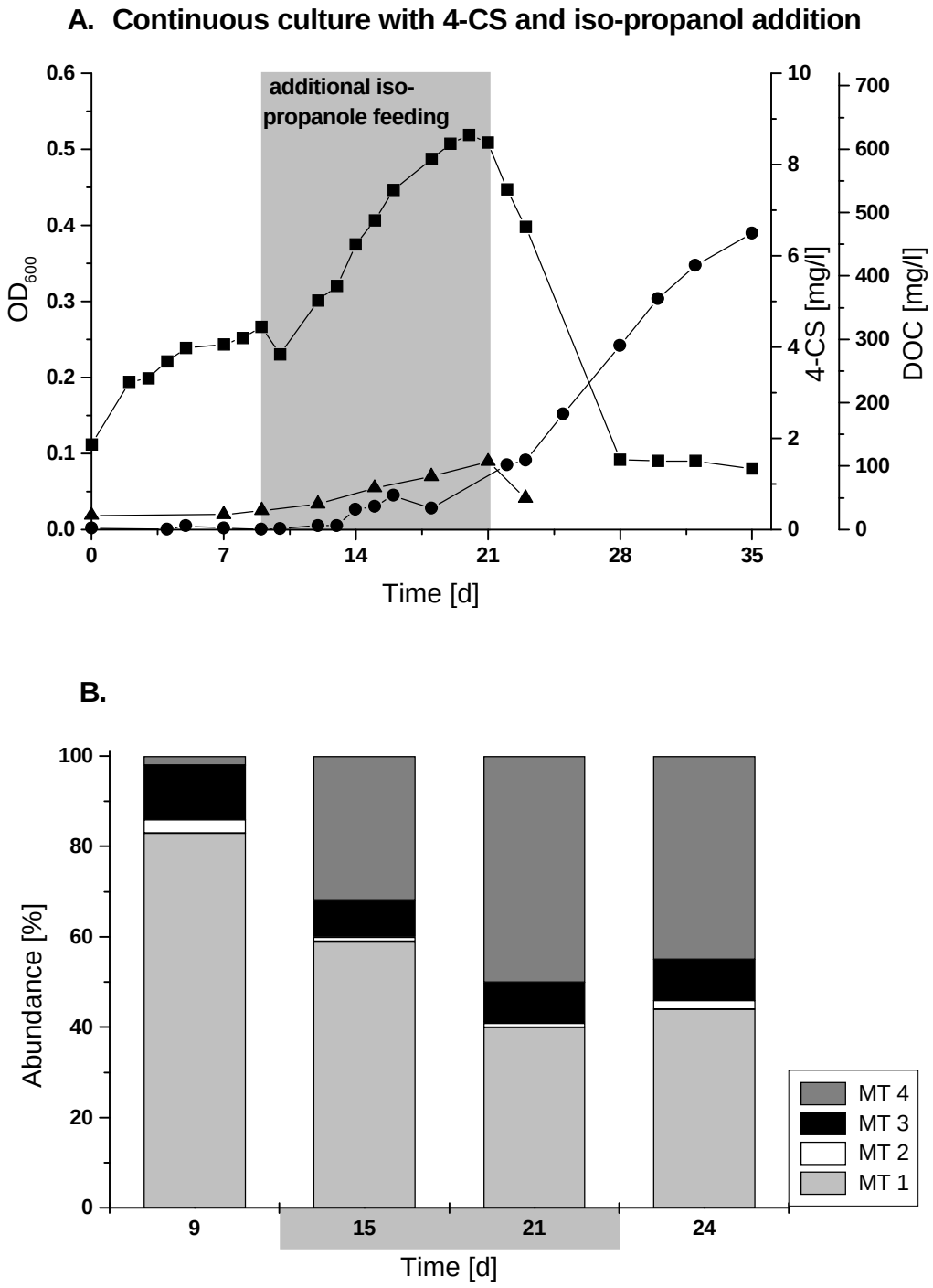

Figure 57: Consortium disturbed by additional feeding of 10 mM iso-propanol; A. Cell number (■), 4-CS concentration (●), and DOC (▲); B. relative abundance of consortium members;

6.3 Addition of competitors to consortium in continuous culture

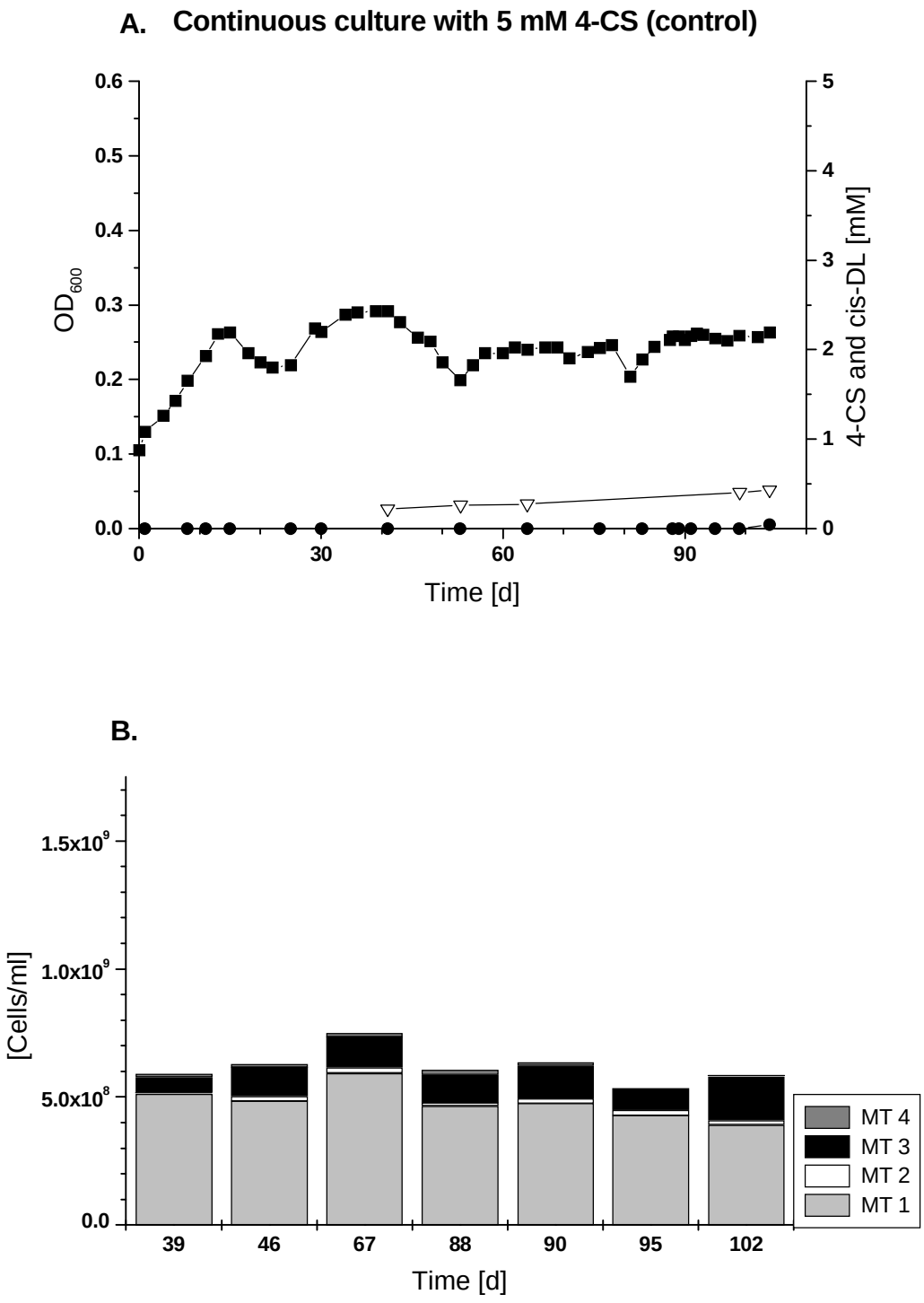

Figure 58: Continuous culture of consortium, growing with 4-CS, undisturbed control; A. Cell number (■), 4-CS (●) and *cis*-DL (▽) concentration, B. absolute abundance of consortium members

6.4 Literature

Adams, R. H., Huang, C. M., Higson, F. K., Brenner, V. & Focht, D. D. (1992). Construction of a 3-chlorobiphenyl-utilizing recombinant from an intergeneric mating. *Applied and environmental microbiology* **58**, 647-654.

Alexander, M. (1997). Microbial communities and interaction: a prelude. In *Manual of environmental microbiology*, pp. 5-13. Edited by C. J. Hurst. Washington: American Society for Microbiology.

Amann, R. I., Krumholz, L. & Stahl, D. A. (1990). Fluorescent-Oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *Journal of bacteriology* **172**, 762-770.

Amann, R. I., Ludwig, W. & Schleifer, K.-H. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiological reviews* **59**, 143-169.

Ambujom, S. (2001). Studies on composition and stability of a large membered bacterial consortium degrading phenol. *Microbiological research* **156**, 293-301.

Arfmann, H.-A., Timmis, K. N. & Wittich, R.-M. (1997). Mineralization of 4-chlorodibenzofuran by a consortium consisting of *Sphingomonas* sp. strain RW1 and *Burkholderia* sp. strain JWS. *Applied and environmental microbiology* **63**, 3458-3462.

Arvin, E., Jensen, B. K. & Gundersen, A. T. (1989). Substrate interactions during aerobic biodegradation of benzene. *Applied and environmental microbiology* **55**, 3221-3225.

Assmus, B., Schloter, M., Kirchhof, G., Hutzler, P. & Hartmann, A. (1997). Improved in situ tracking of rhizosphere bacteria using dual staining with fluorescence-labeled antibodies and rRNA-targeted oligonucleotides. *Microbial ecology* **33**, 32-40.

Asturias, J. A. & Timmis, K. N. (1993). Tree different 2,3-dihydroxybiphenyl-1,2-dioxygenase genes in the gram-positive polychlorobiphenyl-degrading bacterium *Rhodococcus globerulus* P6. *Journal of bacteriology* **175**, 4631-4640.

Atkinson, B. & Mavituna, F. (1991). *Biochemical engineering and biotechnology handbook*, second edition edn. New York: Stockton Press.

Atlas, R. M. (1984). Use of microbial diversity measurements to assess environmental stress. *Current perspectives in microbial ecology*, 540-545.

Atlas, R. M. & Unterman, R. (1999). Bioremediation. In *Manual of industrial microbiology and biotechnology*, pp. 666-681. Edited by A. L. Demain & J. E. Davies. Washington, D.C.: ASM Press.

Baath, E., Díaz-Ravina, M., Frostegard, A. & Campbell, C. D. (1998). Effect of metal-rich sudge amendments on the soil microbial community. *Applied and environmental microbiology* **64**, 238-245.

Babu, K. S., Ajith-Kumar, P. V. & Kunhi, A. A. M. (1995). Simultaneous degradation of 3-chlorobenzoate and phenolic compounds by a defined mixed culture of *Pseudomonas* spp. *World Journal of Microbiology & Biotechnology* **11**, 148-152.

Bailey, J. E. & Ollis, D. F. (1986). Kinetics of substrate utilization, product formation, and biomass production in cell culture. In *Biochemical engineering fundamentals*, pp. 373-456. Edited by V. Kiran & C. C. Martin. New York: McGraw-Hill.

Bailey, M. J., Lilley, A. K., Thompson, I. P., De Leij, F. A. A. M., Whipps, J. M. & Lynch, J. M. (1999). Deliberate Release of Recombinant Microorganisms. In *Manual of industrial microbiology and biotechnology*, pp. 693-703. Edited by A. L. Demain & J. E. Davies. Washington, D.C.: ASM Press.

Balfanz, J. & Rehm, H. J. (1991). Biodegradation of 4-chlorophenol by adsorptive immobilized *Alcaligenes* sp. A 7-2 in soil. *Applied microbiology and biotechnology* **35**, 662-668.

Barles, R. W., Daughton, G. C. & Hsieh, D. P. H. (1979). Accelerated parathion degradation in soil inoculated with aclimated bacteria under field conditions. *Archives of environmental contamination and toxicology* **8**, 647-660.

Bartels, I., Knackmuss, H. J. & Reineke*, W. (1984). Suicide inactivation of catechol 2,3-dioxygenase from *Pseudomonas putida* mt-2 by 3-halocatechols. *Applied and environmental microbiology* **47**, 500-505.

Bauer, J. E. & Capone, D. G. (1988). Effects of co-occurring aromatic hydrocarbons on degradation of individual polycyclic aromatic hydrocarbons in marine sediment slurries. *Applied and environmental microbiology* **54**, 1649-1655.

Blasco, R., Mallavarapu, M., Wittich, R.-M., Timmis, K. N. & Pieper, D. H. (1997). Evidence that formation of protoanemonin from metabolites of 4-chlorobiphenyl degradation negatively affects the survival of 4-chlorobiphenyl-cometabolizing microorganisms. *Applied and environmental microbiology* **63**, 427-434.

Blasco, R., Wittich, R.-M., Mallavarapu, M., Timmis, K. N. & Pieper, D. H. (1995). From xenobiotic to antibiotic, formation of protoanemonin from 4-chlorocatechol by enzymes of the 3-oxoadipate pathway. *Journal of biological chemistry* **49**, 29229-29235.

Boetius, A., Ravenschlag, K., Schubert, C. J., Rickert, D., Widdel, F., Gieseke, A., Amann, R., JOrgensen, B. B., Witte, U. & Pfannkuche, O. (2000). A marine microbial consortium apparently mediating anaerobic oxidation of methane. *Nature* **407**, 623-626.

Bogan, B. W., Lahner, L. M., Trbovic, V., Szajkovics, A. M. & Paterek, J. R. (2001). Effects of Alkylphosphates and Nitrous Oxide on Microbial Degradation of Polycyclic Aromatic Hydrocarbons. *Applied and environmental microbiology* **67**, 2139-2144.

Boon, N., Goris, J., De Vos, P., Verstraete, W. & Top, E. M. (2000). Bioaugmentation of Activated Sludge by an Indigenous 3-Chloroaniline-Degrading *Comamonas testosteroni* Strain, I2*gfp*. *Applied and environmental microbiology* **66**, 2906-2913.

Boronin, A. M. (1991). Genetics of biodegradation. *Acta Biotechnology* **11**, 177-181.

Boyle, M. (1992). The importance of genetic exchange in degradation of xenobiotic chemicals. In *Environmental microbiology*, pp. 319-333. Edited by R. Mitchell. New York: Wiley-Liss.

Bradshaw, D. J., Marsh, P. D., Allison, C. & Schilling, K. M. (1996a). Effect of oxygen, inoculum composition and flow rate on development of mixed-culture oral biofilms. *Microbiology* **142**, 623-629.

Brettar, I., Ramos-Gonzalez, M. I., Ramos, J. L. & Höfle, M. G. (1994). Fate of *Pseudomonas putida* after release into lake water mesocosms: Different survival mechanisms in response to environmental conditions. *Microbial ecology* **27**, 99-122.

Brill, W. J. (1985). Safety concerns and genetic engineering in agriculture. *Science* **227**, 381-384.

Brock, T. D. (1987). The study of microorganisms *in situ:* progress and problems. In *Ecology of microbial communities*, pp. 1-17. Edited by M. Fletcher, T. R. G. Gray & J. G. Jones. Cambridge: Cambridge University Press.

Brückmann, M., Blasco, R., Timmis, K. N. & Pieper, D. H. (1998). Detoxification of protoanemonin by dienelactone hydrolase. *Journal of bacteriology* **180**, 400-402.

Bryant, M. P., Wolin, E. A., Wolin, M. J. & Wolfe, R. S. (1967). *Methanobacillus omelianskii*, a symbiotic association of two species of bacteria. *Archiv für Mikrobiologie* **59**, 20-31.

Bundy, J. G., Paton, G. I. & Campbell, C. D. (2002). Microbial commuities in different soil types do not converge after diesel contamination. *Journal of applied microbiology* **92**, 276-288.

Busse, H.-J., Denner, E. B. M. & Lubitz, W. (1996). Classification and identification of bacteria: current approaches to an old problem. Overview of methods used in bacterial systematics. *Journal of biotechnology* **47**, 3-38.

Caldwell, D. E., Wolfaardt, G. M., Korber, D. R. & Lawrence, J. R. (1997). Cultivation of microbial consortia and communities. In *Manual of environmental microbiology*, pp. 79-90. Edited by C. J. Hurst, G. R. Knudsen, M. J. McInerney, L. D. Stetzenbach & M. W. Walter. Washington, D.C.: American Society for Microbiology.

Carvalho, M. F., Alves, C. C. T., Ferreira, M. I. M., De Marco, P. & Castro, P. M. L. (2002). Isolation and initial characterization of a bacterial consortium able to mineralize fluorobenzene. *Applied and environmental microbiology* **68**, 102-105.

Chapin III, F. S., Walker, B. H., Hobbs, R. J., Hooper, D. U., Lawton, J. H., Sala, O. E. & Tilmann, D. (1997). Biotic control over the functioning of ecosystems. *Science* **277**, 500-504.

Chatterjee, D. K., Kellogg, S. T., Hamada, S. & Chakrabarty, A. M. (1981). Plasmid specifying total degradation of 3-chlorobenzoate ba a modified *ortho* pathway. *Journal of bacteriology* **146**, 639-646.

Chian, S. K. & Mateles, R. I. (1968). Growth of mixed cultures on mixed substrates I. Continuous culture. *Applied microbiology* **16**, 1337-1342.

Christensen, B. B., Haagensen, J. A. J., Heydorn, A. & Molin, S. (2002). Metabolic commensalism and competition in a two-species microbial consortium. *Applied and environmental microbiology* **68**, 2495-2502.

Collins, L. D. & Daugulis, A. J. (1997). Characterization and optimization of a two-phase partitioning bioreactor for the biodegradation of phenol. *Applied microbiology and biotechnology* **48**, 18-22.

Commandeur, L. C. M. & Parsons, J. R. (1994). Biodegradation of halogenated aromatic compounds. In *Biochemistry of microbial degradation*, pp. 423-458. Edited by C. Ratledge. Dordrecht, The Netherlands: Kluwer Academic Publishers.

de Macario, E. C., Macario, A. J. L. & Wolin, M. J. (1982). Specific antisera and immunological procedures for characterization of methanogenic bacteria. *Journal of bacteriology* **149**, 320-328.

De Souza, M. L., Newcombe, D., Alvey, S., Crowley, D. E., Hay, A., Sadowsky, M. J. & Wackett, L. P. (1998). Molecular basis of a bacterial consortium: Interspecies catabolism of Atrazine. *Applied and environmental microbiology* **64**, 178-184.

Didry, N., Dubreuil, L. & Pincas, M. (1991). Activité antibacterienne des vapeurs de protoanémonine. *Pharmazie (franz.)* **46**, 546-547.

Doran, P. M. (1998). *Bioprocess Engineering Principles*. London: Academic Press Limited.

Dorn, E., Hellwig, M., Reineke, W. & Knackmuss, H. J. (1974). Isolation and characterization of a 3-chlorobenzoate degrading Pseudomonad. *Archives of microbiology* **99**, 61-70.

Dorn, E. & Knackmuss, H. J. (1978a). Chemical structure and biodegradability of halogenated aromatic compounds. Two catechol 1,2-dioxygenases from a 3-chlorobenzoate-grown pseudomonad. *Biochemical journal* **174**, 73-84.

Dorn, E. & Knackmuss, H. J. (1978b). Chemical structure and biodegradability of halogenated aromatic compounds. Substituent effects on 1,2-dioxygenation of catechol. *Biochemical journal* **174**, 85-94.

Doyle, J. D., Stotzky, G., McClung, G. & Hendricks, C. W. (1995). Effects of genetically engineered microorganisms on microbial populations and processes in natural habitats. *Advances in applied microbiology* **40**, 237-287.

Duetz, W. A., DE Jong, C., Williams, P. A. & van Andel, J. G. (1994). Competition in chemostat culture between *Pseudomonas* strains that use different pathways for the degradation of toluene. *Applied and environmental microbiology* **60**, 2858-2863.

Duetz, W. A., Marqués, S., Wind, B., Ramos, J. L. & Andel, J. G. (1996). Catabolite repression of the toluene degradation pathway in *Pseudomonas putida* harboring pWW0 under various conditions of nutrient limitation in chemostat culture. *Applied and environmental microbiology* **62**, 601-606.

Dunn, N. W. & Gunsalus, I. C. (1973). Transmissible plasmid coding early enzymes of naphthalene oxidation in *Pseudomonas putida*. *Journal of Bacteriology*, 974-979.

Dykhuizen, D. E. & Davies, M. (1980). An experimental model: bacterial specialists and generalists competing in chemostats. *Ecology* **61**, 876-882.

Egli, T. (1995). The ecological and physiological significance of the growth of heterotrophic microorganisms with mixtures of substrates. *Advances in Microbial Ecology* **14**, 305-386.

Eichner, C. A., Erb, R. W., Timmis, K. N. & Wagner-Döbler, I. (1999). Thermal gradient gel electrophoresis analysis of bioprotection from pollutant shocks in the activated sludge microbial community. *Applied and environmental microbiology* **65**, 102-109.

Engelen, B., Meinken, K., von Wintzingerode, F., Heuer, H., Malkomes, H.-P. & Backhaus, H. (1998). Monitoring impact of a pesticide treatment on bacterial soil communities by metabolic and genetic fingerprinting in addition to conventional testing procedures. *Applied and environmental microbiology* **64**, 2814-2821.

Erb, R. W., Eichner, C. A., Wagner-Döbler, I. & Timmis, K. N. (1997). Bioprotection of microbial communities from toxic phenol mixtures by a genetically designed pseudomonad. *Nature biotechnology* **15**, 378-382.

Farrell, A. & Quilty, B. (1999). Degradation of mono-chlorophenols by a mixed microbial community via a *meta*- cleavage pathway. *Biodegradation* **10**, 353-362.

Faude, U. C. (1996). Immunochemische Analyse mikrobieller Lebensgemeinschaften. In *Fakultät für Biowissenschaften, Pharmazie und Psychologie*, pp. 128. Leipzig: Universität Leipzig.

Faude, U. C. & Höfle, M. G. (1997). Development and application of monoclonal antibodies for in situ detection of indigneous bacterial strains in aquatic ecosystems. *Applied and environmental microbiology* **63**, 4534-4542.

Fava, F., Di Gioia, D., Romagnoli, C., Marchetti, L. & Mares, D. (1993). Biosynthesis and cytoplasmic accumulation of a chlorinated catechol pigment during 3-chlorobenzoate aerobic co-metabolism in *Pseudomonas fluorescens*. *Archives of microbiology* **160**, 350-357.

Feigel, B. J. & Knackmuss, H. J. (1993). Syntrophic interactions during degradation of 4-aminobenzenesulfonic acid by a two species bacterial culture. *Archives of microbiology* **159**, 124-130.

Fernandez, A. S., Hashsham, S. A., Dollhopf, S. L., Raskin, L., Glagoleva, O., Dazzo, F. B., Hickey, R. F., Criddle, C. S. & Tiedje, J. M. (2000). Flexible community structure correlates with stable community function in methanogenic bioreactor communities perturbed by glucose. *Applied and environmental microbiology* **66**, 4058-4067.

Fey, A. & Conrad, R. (2000). Effect of temperature on carbon and electron flow and on the archaeal community in methanogenic rice field soil. *Applied and environmental microbiology* **66**, 4790-4797.

Filonov, A. E., Duetz, W. A., Karpov, A. V., Gaiazov, R. R., Kosheleva, I. A., Breure, A. M., Filonova, I. F., van Andel, J. G. & Boronin, A. M. (1997). Competition of plasmid-bearing *Pseudomonas putida* strains catabolizing naphthalene via various pathways in chemostat culture. *Applied microbiology and biotechnology* **48**, 493-498.

Filonov, A. E., Puntus, I. F., Karpov, A. V., Gaiazov, R. R., Kosheleva, I. A. & Boronin, A. M. (1999). Growth and survival of *Pseudomonas putida* strains degrading naphtalene in soil model systems with different moisture levels. *Process Biochemistry* **34**, 303-308.

Fogarty, A. M. & Tuovinen, O. H. (1995). Microbial degradation of the herbicide dicamba. *Journal of industrial microbiology* **14**, 365-370.

Ford, J. (1989). The effects of chemical stress on aquatic species composition and community structure. In *Ecotoxicology: problems and approaches*. Edited by S. A. Levin, M. A. Harwell, J. R. Kelly & K. D. Kimball. New York: Springer.

Franklin, R. B., Garland, J. L., Bolster, C. H. & Mills, A. L. (2001). Impact of dilution on microbial community structure and functional potential: Comparison of numerical simulations and batch culture experiments. *Applied and environmental microbiology* **67**, 702-712.

Frech, G. (1996). Struktur und Dynamik eines 4-Chlorsalicylat abbauenden Chemostat-Konsortiums. In *Mikrobiologie*, pp. 88. Braunschweig: Universität Carolo-Wilhelmina.

Fuller, M. E., Mailloux, B. J., Zhang, P. F., Streger, S. H., Hall, J. A., Vainberg, S. N., Beavis, A. J., Johnson, W. P., Onstott, T. C. & DeFlaun, M. F. (2001). Field-scale evaluation of CFDA/SE staining coupled with multiple detection methods for assessing the transport of bacteria in situ. *FEMS Microbiology Ecology* **37**, 55-66.

Garland, J. L. (1997). Analysis and interpretation of communitiy-level physiological profiles in microbial ecology. *FEMS microbiology ecology* **24**, 289-300.

Garland, J. L. & Lehmann, R. M. (1999). Dilution/extinction of community phenotypic characters to estimate relative structural diversity in mixed communities. *FEMS microbiology ecology* **30**, 333-343.

Gearing, J. N. (1989). Aquatic microcosms. In *Ecotoxicology: problems and approaches*, pp. 411-470. Edited by S. A. Levin, M. A. Harwell, J. R. Kelly & K. D. Kimball. New York: Springer.

Ghiorse, W. C. & Wilson, J. T. (1988). Microbial ecology of the terrestrial subsurface. *Advances in applied microbiology* **33**, 107-172.

Goldstein, R., Mallory, L. M. & Alexander, M. (1985). Reasons for possible failure of inoculation to enhance biodegradation. *Applied and environmental microbiology* **50**, 977-983.

Gottschal, J. C. (1983). Mixed substrate utilization by mixed cultures. In *Bacteria in Nature*, pp. 261-292. Edited by J. S. Poindexter & E. R. Leadbetter. New York: Plenum Press.

Greenberg, A. E. (1965). Standard methods for the examination of water and wastewater., pp. 1100: American public health association.

Hack, C. D., Woodley, J. M., Lilly, M. D. & Liddel, J. M. (1994). The production of *Pseudomonas putida* for the hydroxylation of toluene fo its cis-glycol. *Applied microbiology and biotechnology* **41**, 495-499.

Hahn, M. W. & Höfle, M. G. (1998). Grazing pressure by a bacterivorous flagellate reverses the relative abundance of *Commamonas acidovorans* PX54 and *Vibrio* strain CB5 in chemostat cocultures. *Applied and environmental microbiology* **64**, 1910-1918.

Halden, R. U., Tepp, S. M., Halden, B. G. & Dwyer, D. F. (1999). Degradation of 3-phenoxybenzoic acid in soil by *Pseudomonas pseudoalcaligenes* POB310(pPOB) and two modified *Pseudomonas* strains. *Applied and environmental microbiology* **65**, 3354-3359.

Haller, H. D. & Finn, R. K. (1979). Biodegradation of 3-chlorobenzoate and formation of black colour in the presence and absence of benzoate. *European journal of applied microbiology* **8**, 191-205.

Harder, W. & Dijkhuizen, L. (1982). Strategies of mixed substrate utilization in microorganisms. *Phil Trans R Soc Lond B* **297**, 459-480.

Harkness, M. R., McDermott, J. B., Abramowicz, D. A., Salvo, J. J., Flanagan, W. P., Stephens, M. L., Mondello, F. J., May, R. J., Lobos, J. H., Carroll, K. M., Brennan, M. J., Bracco, A. A., Fish, K. M., Warner, G. L., Wilson, P. R., Dietrich, D. K., Lin, D. T., Morgan, C. B. & Gately, W. L. (1993). In situ stimulation of aerobic PCB biodegradation in Hudson river sediments. *Science* **259**, 503-507.

Harrison, D. E. F. (1978). Mixed cultures in industrial fermentation processes. *Advances in applied microbiology* **24**, 129-164.

Hartmann, J., Reineke, W. & Knackmuss, H. J. (1979). Metabolism of 3-chloro-, 4-chloro, and 3,5-Dichlorobenzoate by a Pseudomonad. *Applied and environmental microbiology* **37**, 421-428.

Hashsham, S. A., Fernandez, A. S., Dollhopf, S. L., Dazzo, F. B., Hickey, R. F., Tiedje, J. M. & Criddle, C. S. (2000). Parallel processing of substrate correlates with greater functional stability in methanogenic bioreactor communities perturbed by glucose. *Applied and environmental microbiology* **66**, 4050-4057.

Hock, B. (1996). Advances in immunochemical detection of microorganisms. *Ann Biol Clin* **54**, 243-252.

Höfle, M. (1976). Untersuchungen über den Einfluß der Temperatur auf Wachstum und Substratausnutzung heterotropher Gewässerbakterien. In *Fakultät für Biologie*, Freiburg i. Br.: Albert-Ludwigs-Universität.

Hollender, J., Hopp, J. & Dott, W. (1997). Degradation of 4-chlorophenol via the meta cleavage pathway by *Comamonas testosteroni* JH5. *Applied and environmental microbiology* **63**, 4567-4572.

Hopkins, G. D., Semprini, L. & McCarty, P. L. (1993). Microcosm and *in situ* field studies of enhanced biotransformation of trichloroethylene by phenol-utilising microorganisms. *Applied and environmental microbiology* **59**, 2277-2285.

Horvath, R. S. (1972). Microbial co-metabolism and the degradation of organic compounds in nature. *Bacteriological reviews* **36**, 146-155.

Huertas, M.-J., Duque, E., Marqués, S. & Ramos, J. L. (1998). Survival in soil of different toluene-degrading *Pseudomonas* strains after solvent shock. *Applied and environmental microbiology* **64**, 38-42.

Hurst, C. J. (1997). Manual of environmental microbiology. Washington DC: American Society for Microbiology.

Hutchinson, D. H. & Robinson, C. W. (1988). Kinetics of the simultaneous batch degradation of p-cresol and phenol by *Pseudomonas putida*. *Applied microbiology and biotechnology* **29**, 599-604.

Jakobs, B. (1997). Entwicklung von Gen-Kassetten, weche für Enzyme des Chlorbrenzkatechinabbaus kodierende Gene enthalten und deren Nutzung zur Erweiterung des Substratspektrums verschiedener Rezipientenstämme. In *Naturwissenschaftliche Fakultät*, pp. 165. Braunschweig: Technische Universität Carolo-Wilhelmina.

Ka, J. O., Holben, W. E. & Tiedje, J. M. (1994). Analysis of competition in soil among 2,4-dichlorophenoxyacetic acid-degrading bacteria. *Applied and environmental microbiology* **60**, 1121-1128.

Kapley, A., Tolmare, A. & Purohit, H. J. (2001). Role of oxygen in the utilization of phenol by *Pseudomonas* CF600 in continuous culture. *World Journal of Microbiology & Biotechnology* **17**, 801-804.

Kaschabek, S. R. & Reineke, W. (1995). Maleylacetate reductase of *Pseudomonas* sp. strain B13: specificity of substrate conversion and halide elimination. *Journal of bacteriology* **177**, 320-325.

Kästner, M., Breuer-Jammali, M. & Mahro, B. (1998). Impact of inoculation protocols, salinity, and pH on the degradation of polycyclic aromatic hydrocarbons (PAHs) and survival of PAH-degrading bacteria introduced into soil. *Applied and environmental microbiology* **64**, 359-362.

Kell, D. B., Kaprelyants, A. S., Weichart, D. H., Harwood, C. R. & Barer, M. R. (1998). Viability and activity in radily culturable bacteria: a review and discussion of the practical issues. *Antonie van Leeuwenhoek* **73**, 169-187.

Kim, S. & Picardal, F. (2001). Microbial growth on dichlorobiphenyls chlorinated on both rings as a sole carbon and energy source. *Applied and environmental microbiology* **67**, 1953-1955.

Klemba, M., Jakobs, B., Wittich, R. M. & Pieper, D. (2000). Chromosomal integration of *tcb* chlorocatechol degradation pathway genes as a means of expanding the growth substrate range of bacteria to include haloaromatics. *Applied and environmental microbiology* **66**, 3255-3261.

Koch, A. L. (1961). Some calculations on the turbidity of mitrochondria and bacteria. *Biochimica et biophysica acta* **51**, 429-441.

Koch, A. L. (1997). Microbial physiology and ecology of slow growth. *Microbiology and Molecular Biology Reviews* **61**, 305-318.

Konopka, A., Oliver, L. & Turco, R. F. (1998). The use of carbon substrate utilization patterns in environmental and ecological microbiology. *Microbial ecology* **35**, 103-115.

Krooneman, J., Wieringa, E. B. A., Moore, E. R. B., Gerritse, J., Prins, R. A. & Gottschal, J. C. (1996). Isolation of *Alcaligenes* sp. strain L6 at low oxygen concentrations and degradation of 3-chlorobenzoate via a pathway not involving (chloro)catechols. *Applied and environmental microbiology* **62**, 2427-2434.

Lay, J. J., Li, Y. Y., Noike, T., Endo, J. & Ishimoto, S. (1997). Analysis of environmental factors affecting methane production from high-solids organic waste. *Wat. Sci. Tech.* **36**, 493-500.

Lehrbach, P. R., Zeyer, J., Reineke, W., Knackmuss, H.-J. & Timmis, K. N. (1984). Enzyme recruitment in vitro: Use of cloned genes to extend the range of haloaromatics degraded by *Pseudomonas* sp. strain B 13. *Journal of bacteriology* **158**, 1025-1032.

Lendenmann, U., Snozzi, M. & Egli, T. (1996). Kinetics of the simultaneous utilization of sugar mixtures by *Escherichia coli* in continuous culture. *Applied and environmental microbiology* **62**, 1493-1499.

Lenski, R. E. (1993). Evaluating the fate of genetically modified microorganisms in the environment: are they inherently less fit? *Experientia* **49**, 201-209.

Lister, D. L., Sproule, R. F., Britt, A. J., Lowe, C. R. & Bruce, N. C. (1996). Degradation of cocaine by a mixed culture of *Pseudomonas fluorescens* MBER and *Comamonas acidovorans* MBLF. *Applied and environmental microbiology* **62**, 94-99.

Magliette, R. J., McKinney, D. & Venkat, E. (1996). Acclimation and selection for methanesulfonic acid (MSA) degrading microbial population using continuous culture techniques. *Bull Environ Contam Toxicol* **57**, 406-12.

Mars, A. E., Kasberg, T., Kaschabek, S. R., van Agteren, M. H., Janssen, D. B. & Reineke, W. (1997). Microbial degradation of chloroaromatics: use of the meta-cleavage pathway for mineralization of chlorobenzene. *Journal of bacteriology* **179**, 4530-4537.

McClure, N. C., Fry, J. C. & Weightman, A. J. (1991). Survival and catabolic activity of natural and genetically engineered bacteria in a laboratory-scale activated-sludge unit. *Applied and environmental microbiology* **57**, 366-373.

McFeters, G. A., Yu, F. P., Pyle, B. H. & Stewart, P. S. (1995). Phyisological assessment of bacteria using fluorochromes. *Journal of microbiological methods* **21**, 1-13.

Morales, A., Garland, J. L. & Lim, D. V. (1996). Survival of potentially pathogenic human-associated bacteria in the rhizosphere of hydroponically-grown wheat. *FEMS microbiology ecology* **20**, 155-162.

Morris, C. M. & Barnsley, E. A. (1982). The cometabolism of 1- and 2-chloronaphthalene by pseudomonads. *Canadian journal of microbiology* **28**, 73-79.

Moser, A. (1985). Kinetics of batch fermentations. In *Biotechnology*, pp. 243-283. Edited by H. J. Rehm, G. Reed & H. Brauer. Weinheim: VCH Verlagsgesellschaft.

Müller, R., Deckwer, W.-D. & Hecht, V. (1996). Degradation of chloro- and methyl-substituted benzoic acids by a genetically modified microorganism. *Biotechnology and bioengineering* **51**, 528-537.

Naeem, S., Thompson, L. J., Lawler, S. P., Lawton, J. H. & Woodfin, R. M. (1994). Declining biodiversity can alter the performance of ecosystems. *Nature* **368**, 734-737.

Nielsen, A. T., Tolker-Nielsen, T., Barken, K. B. & Molin, S. (2000). Role of commensal relationships on the spatial structure of a surface-attached microbial cinsortium. *Environmental Microbiology* **2**, 59-68.

Nikodem, P., Hecht, V., Schlömann, M. & Pieper, D. H. (2003). New bacterial pathway for 4- and 5-chlorosalicylate degradation via 4-chlorocatechol and maleylacetate in *Pseudomonas* sp. strain MT 1. *Journal of bacteriology* **185**, 6790-6800.

Nusslein, K., Maris, D., Timmis, K. & Dwyer, D. F. (1992). Expression and transfer of engineered catabolic pathways harbored by *Pseudomonas* spp. introduced into activated sludge microcosms. *Applied and environmental microbiology* **58**, 3380-3386.

Ogram, A. & Feng, H. (1997). Methods of soil microbial community analysis. In *Manual of environmental microbiology*, pp. 422-430. Edited by C. J. Hurst, G. R. Knudsen, M. J. McInerney, L. D. Stetzenbach & M. W. Walter. Washington, D.C.: ASM Press.

Olsen, R. H., Kukor, J. J., Byrne, A. M. & Johnson, G. R. (1997). Evidence for the evolution of a single component phenol/cresol hydroxylase from a multicomponent toluene monooxygenase. *Journal of industrial microbiology and biotechnology* **19**, 360-368.

Owens, J. D. & Legan, J. D. (1987). Determination of the monod substrate saturation constant for microbial growth. *FEMS microbiology reviews* **46**, 419-432.

Paerl, H. W. (1998). Structure and function of anthropogenically altered microbial communities in coastal waters. *Current opinion in microbiology* **1**, 296-302.

Pelz, O. (1999a). Functional characterisation of a 4-chlorosalicylate-degrading bacterial community by δ^{13} labeling of biomarkers. In *Naturwissenschaftliche Fakultät*, pp. 114. Braunschweig: Technischen Universität Carolo-Wilhelmina.

Pelz, O., Tesar, M., Wittich, R.-M., Moore, E. R. B., Timmis, K. N. & Abraham, W.-R. (1999b). Towards elucidation of microbial community metabolic pathways: unraveling the network of carbon sharing in a pollutant degrading bacterial consortium by immunocapture and isotopic ratio mass spectrometry. *Environmental Microbiology* **1**, 167-174.

Pfeil, K. M. & Gaudy, A. F. (1971). Kinetic constants for aerobic growth of microbial populations selected with various single compounds and with municipal wastes as substrates. *Applied microbiology* **21**, 253-256.

Pfennig, N. (1980). Syntrophic mixed cultures and symbiotic consortia with phototrophic bacteria: a review. In *Anaerobes and anaerobic infections*, pp. 127-131. Edited by G. Gottschalk, N. Pfennig & H. Werner. Stuttgart: Gustav Fischer Verlag.

Philips, O. M. (1973). The equilibirium and stability of simple marine biological systems I Primary nutrient consumers. *American Naturalist* **107**, 73-93.

Pieper, D. H. & Timmis, K. N. (1996). Vom Xenobiotikum zum Antibiotikum: Ein grundlegendes ökotoxikologisches Problem beim Abbau von Chloraromaten und dessen Lösung durch genetisches Design. In *Wissenschaftlicher Ergebnisbericht der Gesellschaft für Biotechnologische Forschung mbH*, pp. 31-38. Braunschweig.

Pipke, R., Wagner-Döbler, I., Timmis, K. N. & Dwyer, D. F. (1992). Survival and function of a genetically engineered Pseudomonad in aquatic sediment microcosms. *Applied and environmental microbiology* **58**, 1259-1265.

Porter, J., Robinson, J., Pickup, R. & Edwards, C. (1995). Recovery of a bacterial sub-population from sewage using immunofluorescent flow cytometry and cell sorting. *FEMS microbiology letters* **133**, 195-199.

Prichard, P. H. (1992). Use of inocculation in bioremediation. *Current opinion in biotechnology* **3**, 232-243.

Prucha, M., Peterseim, A. & Pieper, D. H. (1997). Evidence for an isomeric muconolactone isomerase involved in the metabolism of 4-methylmuconolactone by *Alcaligenes eutrophus* JMP134. *Archives of microbiology* **168**, 33-38.

Ramos, J. L., Díaz, E., Dowling, D., de Lorenzo, V., Molin, S., O'Gara, F., Ramos, C. & Timmis, K. N. (1994). The behavior of bacteria designed for biodegradation. *Bio-Technology <New York>* **12**, 1349-1356.

Ramos, J. L., Haidour, A., Duque, E., Pinar, G., Calvo, V. & Oliva, J.-M. (1996). Metabolism of nitrate esters by a consortium of two bacteria. *Nature biotechnology* **14**, 320-322.

Ramos-González, M.-I., Ruiz-Cabello, F., Brettar, I., Garrido, F. & Ramos, J. L. (1992). Tracking genetically engineered bacteria: Monoclonal antibodies against surface determinants of the soil bacterium *Pseudomonas putida* 2440. *Journal of bacteriology* **174**, 2978-2985.

Reber, H. H. & Kaiser, P. (1981). Regulation of the utilization of glucose and aromatic substrates in four strains of *Pseudomonas putida*. *Archives of microbiology* **130**, 243-247.

Reineke, W. (1984). Microbial degradation of halogenated aromatic compounds. In *Microbial degradation of organic compounds*, pp. 319-360. Edited by D. T. Gibson. New York: Marcel Dekker.

Reineke, W. (1999). Mikrobieller Abbau von Chloraromaten. In *Biotechnologie im Umweltschutz: Bioremediation: Entwicklungsstand - Anwendungen - Perspektiven*, pp. 64-98. Edited by S. Heiden. Munster: Erich Schmidt Verlag, Berlin.

Reineke, W., Jeenes, D. J., Williams, P. A. & Knackmuss, H. J. (1982). TOL plasmid pWWO in constructed halobenzoate-degrading *Pseudomonas* strains: Prevention of the *meta*-pathway. *Journal of bacteriology* **150**, 195-201.

Reineke, W. & Knackmuss, H. J. (1978). Chemical structure and biodegradability of halogenated aromatic compounds. Substituent effects on 1,2-dioxygenation of benzoic acid. *Biochimica et biophysica acta* **542**, 412-423.

Riegert, U., Heiss, G., Fischer, P. & Stolz, A. (1998). Distal cleavage of 3-chlorocatechol by an extradiol dioxygenase to 3-chloro-2-hydroxymuconic semialdehyde. *Journal of bacteriology* **180**, 2849-2853.

Ringelberg, D. B., Talley, J. W., Perkins, E. J., Tucker, S. G., Luthy, R. G., Bouwer, E. J. & Fredrickson, H. L. (2001). Succession of Phenotypic, Genotypic, and Metabolic Community Characteristics during In Vitro Bioslurry Treatment of Polycyclic Aromatic Hydrocarbon-Contaminated Sediments. *Applied and environmental microbiology* **67**, 1542-1550.

Rogers, J. B., DuTeau, N. M. & Reardon, K. F. (2000). Use of 16S-rRNA to investigate microbial population dynamics during biodegradation of toluene and phenol by a binary culture. *Biotechnology and bioengineering* **70**, 436-445.

Rojo, F., Pieper, D. H., Engesser, K. H., Knackmuss, H. J. & Timmis, K. N. (1987). Assemblage of ortho cleavage route for simultaneous degradation of chloro- and methylaromatics. *Science* **238**, 1395-1398.

Rubio, M. A., Engesser, K.-H. & Knackmuss, H.-J. (1986a). Microbial metabolism of chlorosalicylates: accelerated evolution by natural genetic exchange. *Archives of microbiology* **145**, 116-122.

Rubio, M. A., Engesser, K.-H. & Knackmuss, H.-J. (1986b). Microbial metabolism of chlorosalicylates: effect of prolonged subcultivation on constructed strains. *Archives of microbiology* **145**, 123-125.

Sayler, G. S. & Ripp, S. (2000). Field applications of genetically engineered microorganisms for bioremediation processes. *Current opinion in biotechnology* **11**, 286-289.

Schiefer, G. E. & Caldwell, D. E. (1982). Synergistic interaction between *Anabaena* and *Zoogloea* spp. in carbon dioxide-limited continuous cultures. *Applied and environmental microbiology* **44**, 84-87.

Schimel, J. (1995). Ecosystem consequences of microbial diversity and community structure. In *Ecological studies*, pp. 239-254. Edited by Chapin & Korner. Berlin.

Schmidt, E., Hellwig, M. & Knackmuss, H. J. (1983). Degradation of chlorophenols by a defined mixed microbial community. *Applied and environmental microbiology* **46**, 1038-1044.

Schmidt, S. K. & Alexander, M. (1985). Effects of dissolved organic carbon and second substrates on the biodegradation of organic compounds at low concentrations. *Applied and environmental microbiology* **49**, 822-827.

Schmitz, A., Gartemann, K.-H., Fiedler, J., Grund, E., Denecke, B. & Eichenlaub, R. (1995). Degradation of polycyclic and halogenated aromatic compounds by *Rhodococcus* and *Arthrobacter* species. *Biotechnologiya* **7-8**, 138-142.

Schwieger, F. & Tebbe, C. C. (2000). Effect of field inoculation with *Sinorhizobium meliloti* L33 on the composition of bacterial communities in rhizospheres of a target plant (*Medicago sativa*) and a non-target plant (*Chenopodium album*) - Linking of 16S rRNA gene-based single-strand conformation polymorphism community profiles to the diversity of cultivated bacteria. *Applied and environmental microbiology* **66**, 3556-3565.

Senior, E., Bull, A. T. & Slater, J. H. (1976). Enzyme evolution in a microbial community growing on the herbicide Dalapon. *Nature* **263**, 476-479.

Shiaris, M. P. (1989). Seasonal biotransformation of naphthalene, phenanthrene, and benzo[*a*]pyrene in surficial estuarine sediments. *Applied and environmental microbiology* **55**, 1391-1399.

Shimao, M., Fujita, I., Kato, N. & Sakazawa, C. (1985). Enhancement of pyrroloquinoline quinone production and poyvinyl alcohol degradation in mixed continuous cultures of *Pseudomonas putida* VM15A and *Pseudomonas* sp. strain VM15C with mixed carbon sources. *Applied and environmental microbiology* **49**, 1389-1391.

Slater, J. H. & Lovatt, D. (1984). Biodegradation and the significance of microbial communities. In *Microbial degrdation of organic compounds*, pp. 439-485. Edited by D. T. Gibson. New York: Marcel Dekker.

Stahl, D. A. (1997). Molecular approaches for the measurement of density, diversity, and phylogeny. In *Manual of environmental microbiology*, pp. 102-114. Edited by C. J. Hurst, G. R. Knudsen, M. J. McInerney, L. D. Stetzenbach & M. W. Walter. Washington, D.C.: ASM Press.

Steffensen, W. S. & Alexander, M. (1995). Role of competition for inorganic nutrients in the biodegradation of mixtures of substrates. *Applied and environmental microbiology* **61**, 2859-2862.

Stoffels, M., Amann, R., Ludwig, W., Hekmat, D. & Schleifer, K. H. (1998). Bacterial community dynamics during start-up of a trickle-bed bioreactor degrading aromatic compounds. *Appl Environ Microbiol* **64**, 930-9.

Sung Bae, H., Lee, J. M., Bae Kim, Y. & Lee, S.-T. (1996). Biodegradation of the mixtures of 4-chlorophenol and phenol by *Comamonas testosteroni* CPW301. *Biodegradation* **7**, 463-469.

Swindoll, C. M., Aelion, C. M. & Pfaender, F. K. (1988). Influence of organic nutrients on aerobic biodegradation and on the adaptation response of subsurface microbial communities. *Applied and environmental microbiology* **54**, 212-217.

Tesar, M., Hoch, C., Moore, E. R. B. & Timmis, K. N. (1996). Westprinting: Development of a rapid immunochemical identification for species within the genus *Pseudomonas* sensu stricto. *Systematic and applied microbiology* **19**, 577-588.

Thakur, I. S. (1995). Structural and functional characterization of a stable, 4-chlorosalicylic-acid-degrading, bacterial community in a chemostat. *World journal of Microbiology & Biotechnology* **11**, 643-645.

Timmis, K. N. (1994). Designing microorganisms for the treatment of toxic wastes. *Annual review of microbiology* **48**, 525-557.

Torsvik, V., Goksoyr, J. & Daae, F. L. (1990). High diversity in DNA of soil bacteria. *Applied and environmental microbiology* **56**, 782-787.

Trevors, J. T., Kuikman, P. & van Elsas, J. D. (1994). Release of bacteria into soil: cell numbers and distribution. *Journal of microbiological methods* **19**, 247-259.

Troussellier, M., Courties, C. & Vaquer, A. (1993). Recent applications of flow cytometry in aquatic microbial ecology. *Biol Cell* **78**, 111-121.

van den Wijngaard, A. J., van der Kleij, R. G., Doornweerd, R. E. & Janssen, D. B. (1993). Influence of organic nutrients and cocultures on the competitive behavior of 1,2-dichloroethane-degrading bacteria. *Applied and environmental microbiology* **59**, 3400-3405.

van der Meer, J. R., de Vos, W. M., Harayama, S. & Zehnder, A. J. B. (1992). Molecular mechanisms of genetic adaption to xenobiotic compounds. *Microbiological reviews* **56**, 677-694.

Villiesid, F. & Lilly, M. D. (1992). Influence of dissolved oxygen tension on the synthesis of catechol 1,2-dioxygenase by *Pseudomonas putida*. *Enzyme and microbial technology* **14**, 561-565.

Wagner-Döbler, I., Pipke, R., Timmis, K. N. & Dwyer, D. F. (1992). Evaluation of aquatic sediment microcosms and their use in assessing possible effects of introduced microorganisms on ecosystem parameters. *Applied and environmental microbiology* **58**, 1249-1258.

Wagner-Döbler, I., Wittich, R.-M. & Timmis, K. N. (1993). Der mikrobiologische Schatz im Schlamm., pp. 17-28. Braunschweig: Gesellschaft für Biotechnologische Forschung (GBF).

Watanabe, K., Yamamoto, S., Hino, S. & Harayama, S. (1998). Population dynamics of phenol-degrading bacteria in activated sludge determined by gyrB-targeted quantitative PCR. *Applied and environmental microbiology* **64**, 1203-1209.

Westmeier, F. & Rehm, H. J. (1987). Degradation of 4-chlorophenol in municipal wastewater by adsorptiv immobilized *Alcaligenes* sp. A 7-2. *Applied microbiology and biotechnology* **26**, 78-83.

White, D. C., Pinkart, H. C. & Ringelberg, D. B. (1997). Biomass meassurements: Biochemical approaches. In *Manual of environmental microbiology*, pp. 91-101. Edited by C. J. Hurst, G. R. Knudsen, M. J. McInerney, L. D. Stetzenbach & M. W. Walter. Washington, D.C.: ASM Press.

Wieser, M., Eberspächer, J., Vogler, B. & Lingens, F. (1994). Metabolism of 4-chlorophenol by *Azotobacter* sp. GP1: Structure of the *meta* cleavage product of 4-chlorocatechol. *FEMS microbiology letters* **116**, 73-78.

Wilkes, H., Wittich, R.-M., Timmis, K. N., Fortnagel, P. & Francke, W. (1996). Degradation of chlorinated dibenzofurans and dibenzo-*p*-dioxins by *Sphingomonas* sp. strain RW1. *Applied and environmental microbiology* **62**, 367-371.

Wilson, L. P. & Bouwer, E. J. (1997). Biodegradation of organic compounds under mixed oxygen/denitrifying conditions: a review. *Journal of industrial microbiology and biotechnology* **18**, 116-130.

Wilson, M. & Lindow, S. E. (1993). Release of recombinant microorganisms. *Annual reviews of microbiology* **47**, 913-944.

Wintzingerode, F., Göbel, U. B. & Stackebrandt, E. (1997). Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. *FEMS microbiology reviews* **21**, 213-229.

Wittich, R. M., Strömpl, C., Moore, E. R. B. & Timmis, K. N. (1999). Interaction of *Sphingomonas* and *Pseudomonas* strains in the degradation of chlorinated dibenzofurans. *Journal of industrial microbiology and biotechnology* **23**, 353-358.

Wolfaardt, G. M., Lawrence, J. R., Robarts, R. D. & Caldwell, D. E. (1994). The role of interactions, sessile growth and nutrient amendments on the degradative efficiency of a microbial consortium. *Canadian journal of microbiology* **40**, 331-340.

Yaganita, T. (1990). *Natural microbial communities.* Tokyo and Berlin, Heidelberg, New York: Japan ScientificSocieties Press and Springer-Verlag.

Yen, K.-M. & Gunsalus, I. C. (1982). Plasmid gene organization: Naphthalene/salicylate organisation. *Proceedings of the National Academy of Sciences of the United States of America* **79**, 874-878.

Yerushalmi, L., Lascourreges, J. F., Rhofir, C. & Guiot, S. R. (2001). Detection of intermediate metabolites of benzene biodegradation under microaerophilic conditions. *Biodegradation* **12**, 379-391.

Zaidi, B. R. & Metha, N. K. (1995). Effects of organic compounds on the degradation of *p*-nitrophenol in lake and industrial wastewater by inoculated bacteria. *Biodegradation* **6**, 275-281.

Zeikus, J. G. & Johnson, E. A. (1991). *Mixed culture in biotechnology*. New York: McGraw-Hill.

Zellner, G., Feuerhake, E., Joerdening, H. J., Macario, A. J. L. & Conway de Macario, E. (1995). Denitrifying and methanogenic bacteria in the biofilm of a fixed-film reactor operated with methanol/nitrate demonstrated by immunofluorescence and microscopy. *Applied microbiology and biotechnology.* **43**, 566-571.

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